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Abstract

Our objective is to develop and test proteomic methods for the prediction of breast cancer risk, an approach that has not been attempted previously. Our underlying hypothesis is that proteomic analysis of serum will identify proteins differentially expressed in women who do versus those who do not develop invasive breast cancer, and that these differences will be identifiable prior to the clinical presentation of breast cancer. Our work is being conducted in two phases, a training phase and a test phase. Both phases will be conducted as case-control studies nested in a population-based cohort of women who were members of Kaiser Permanente. These serum specimens were collected between 1986 and 1992. We have finalized our cohort definition, finalized the definition of cases and controls; finalized the criteria for matching controls to cases; selected the cases and controls; pulled and aliquotted the serum specimens. For the proteomic analysis, we have developed a detailed protocol for analysis of the serum samples. Briefly, the serum sample is loaded onto an immunoaffinity column to deplete twelve abundant proteins, and the flow-through fraction is collected and subjected to tryptic digestion. Subsequently, the peptides are labeled with iTRAQ reagents and fractionated by cation exchange chromatography (SCX). Six pooled SCX fractions are separately loaded onto a reverse phase column and followed by MALDI-TOF/TOF (4700 Proteomic Analyzer) analyses. The data collected are automatically processed, combined, and searched against a human protein database. This procedure has been thoroughly tested for reproducibility, quantitation and complexity (dynamic range) and the routine collection of case/control data has been initiated. By applying this high-resolution proteomic approach to a prospective setting, this ongoing project should enhance our ability to identify those women at increased risk of breast cancer and intervene before they progress to cancer. Furthermore, it is expected to provide insight into the biological processes underlying breast cancer development through the identification of protein markers of disease and disease susceptibility genes.

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Introduction

New approaches to breast cancer risk prediction are needed given the modest discriminatory accuracy of existing risk prediction models at the individual level. Our objective is to develop and test proteomic methods for the prediction of breast cancer risk, an approach that has not been attempted previously. Our underlying hypothesis is that proteomic analysis of serum will identify proteins differentially expressed in women who do versus those who do not develop invasive breast cancer, and that these differences will be identifiable prior to the clinical presentation of breast cancer. Specifically: (1) using a training set of serum specimens sampled from a population-based cohort of women who were members of Kaiser Permanente, who had their blood samples taken at a multiphasic health examination (MHC) between 1986 and 1992**,** and who were followed up to determine subsequent breast cancer occurrence, we are applying highly sensitivity proteomic approaches in order to identify biomarkers that discriminate between women who developed invasive breast cancer within 5 years of having a serum sample collected (cases) and women who remained free of breast cancer for at least that long (controls); (2) using a validation set of serum samples collected from a separate group of cases and controls in the cohort, we plan to test the biomarkers identified using the training set. Nested case-control studies will be undertaken at the training (32 cases/32 controls) and validation stages (20 cases/20 controls). Cases will be women with no history of breast cancer at baseline who developed a subsequent incident, invasive breast cancer within 5 years of collection of a serum sample; controls (no breast cancer history) will be selected from the same cohort using risk-set sampling and matched individually to cases on calendar year of the MHC exam at which the serum sample of interest was collected, age at that MHC exam, and time since last meal. To reduce the possible impact of breast cancer heterogeneity on interpretation of the results, we have restricted inclusion to white, postmenopausal women; should the results prove informative, we plan to study other subgroups (e.g., African American, premenopausal, etc.) in the future. Data on breast cancer risk factors will come from questionnaire and physical exam data gathered at the MHC. All proteomic spectra will be analyzed by the Mascot program to identify the protein sequences generating all peptide ions. After biomarker ions have been identified, validation will be carried out by the same analytical procedures following only the biomarker ions of interest. The laboratory work will be performed “blinded” to case-control status. Supervised clustering algorithms such as support vector machines will be used for the statistical analysis of the training dataset to identify candidate marker patterns that best discriminate between cases and controls. The most promising markers identified in the training set will be applied to subjects in the validation set and used to classify them as cases, controls, or neither. In contrast to the training set, where case-control status will be known to the data analysts, case-control status will not be known during analysis of the validation set. Therefore, we will determine how well the method developed at the training stage discriminates between cases and controls in the validation set.

Body

(i) Eligibility criteria

We have finalized eligibility for inclusion in the cohort. We had proposed initially to use a cohort recruited between 1964 and 1971. However, because serum samples from that cohort may previously have undergone thawing and re-freezing, we decided to switch to the more recent Kaiser cohort, which was recruited between 1986 and 1992. None of the samples from the latter cohort have been thawed to date. The cohort consists of white, postmenopausal women aged 55 to 80 years at the time of the blood draw. As indicated in our original proposal, we chose to restrict attention to

white women to reduce the possible impact of breast cancer heterogeneity on interpretation of the results.

(ii) Case/control definition and selection

The work for this project is being conducted as nested case-control studies, both at the training and at the validation stages. Cases are defined as white, postmenopausal women with no history of breast cancer at the time of recruitment. Inclusion is restricted to subjects between the ages of 55 and 80 years. We identified 68 potentially eligible cases by merging data from the multiphasic cohort/serum repository databases with data from the Kaiser Permanente tumor registry. The 60 cases selected for the study are a random sample of all eligible cases. Controls are matched 1:1 to the corresponding case. They are white, postmenopausal women with no history of breast cancer and who have not developed breast cancer by the date of diagnosis of the corresponding case. The controls were selected using risk-set sampling with replacement. They were matched to the corresponding case on age (within 1 year), date of serum collection (to within 1 month), and time since last meal (0-3 hours, 4-9 hours). Furthermore, cases and controls were matched with respect to membership of Kaiser Permanente, in the sense that as with the cases, controls were required to have been members from one year prior to serum collection and to have been a member at the time of diagnosis of the corresponding case (those whose membership lapsed for a period exceeding 3 months ceased to be eligible to be selected as a control).

(iii) Data file

Study identification numbers were assigned to the cases and controls. These numbers are linkable to the Kaiser IDs, which allowed extraction of corresponding covariate information from the cohort database. The data file containing the covariate information required for the analysis of the training set data has been created.

(iv) Aliquoting of serum samples

The pulling, testing (for dessication), and aliquoting of the serum specimens was completed at the Orentreich Foundation. For each subject included in the study, 5 x 20 μl aliquots were made. Furthermore, a common serum pool was created by adding 50 μl from each study subject to the pool. The pool serves as a common standard that is included in each run. For each case-control pair, 5 sets of samples were prepared, each set consisting of 20 μl aliquots for the case, the matched control, and the common pool. The location of the case and control aliquot within each triplet was assigned randomly and the laboratory staff are blinded to the identity of the case and control samples. The specimens were shipped to and received at the Albert Einstein College of Medicine where they are held in storage at -80^{o}C until they are needed for analysis.

(v) Development of methods for proteomic analysis of serum samples

In the previous report, the depletion, labeling, and digestion steps had been worked out, with the strong cation exchange (SCX) and reversed phase-MALDI target spotting routines remaining to be optimized. In addition, the protocol for automated data acquisition required fine-tuning. Despite several technical difficulties, we have now established detailed protocols and reaction conditions for each step of the proteomic analysis. Specifically, we have developed an integrated, highly sensitive, protocol for proteomic analysis involving fractionation by immunodepletion and multi-dimensional HPLC and analysis by MALDI-TOF/TOF-MS, to tackle the complexity and dynamic range of the serum proteome. The sequence of steps in this analysis is summarized in Figure 1 and the detailed description of the procedure is provided in the Proteomic Laboratory Operation Manual (Appendix

1) and the checklists for each step (Appendix 2). Briefly, each serum sample is loaded onto an immunoaffinity column to deplete twelve abundant proteins, and the flow-through fraction is collected and subjected to tryptic digestion. Subsequently, the peptide digest is labeled with iTRAQ reagent, mixed with two other digests (to combine differentially labeled case, control, and pool) and subjected to cation exchange (SCX) chromatography. Each SCX fraction is then loaded onto the reverse phase column and pooled fractions spotted onto plates for MALDI-TOF/TOF (4700 Proteomic Analyzer) analyses. The data collected are automatically processed, combined, and searched against human protein databases. The raw spectral files are processed to provide appropriate bioinformatic data. All stages of this procedure have been developed and refined over the last year as described below.

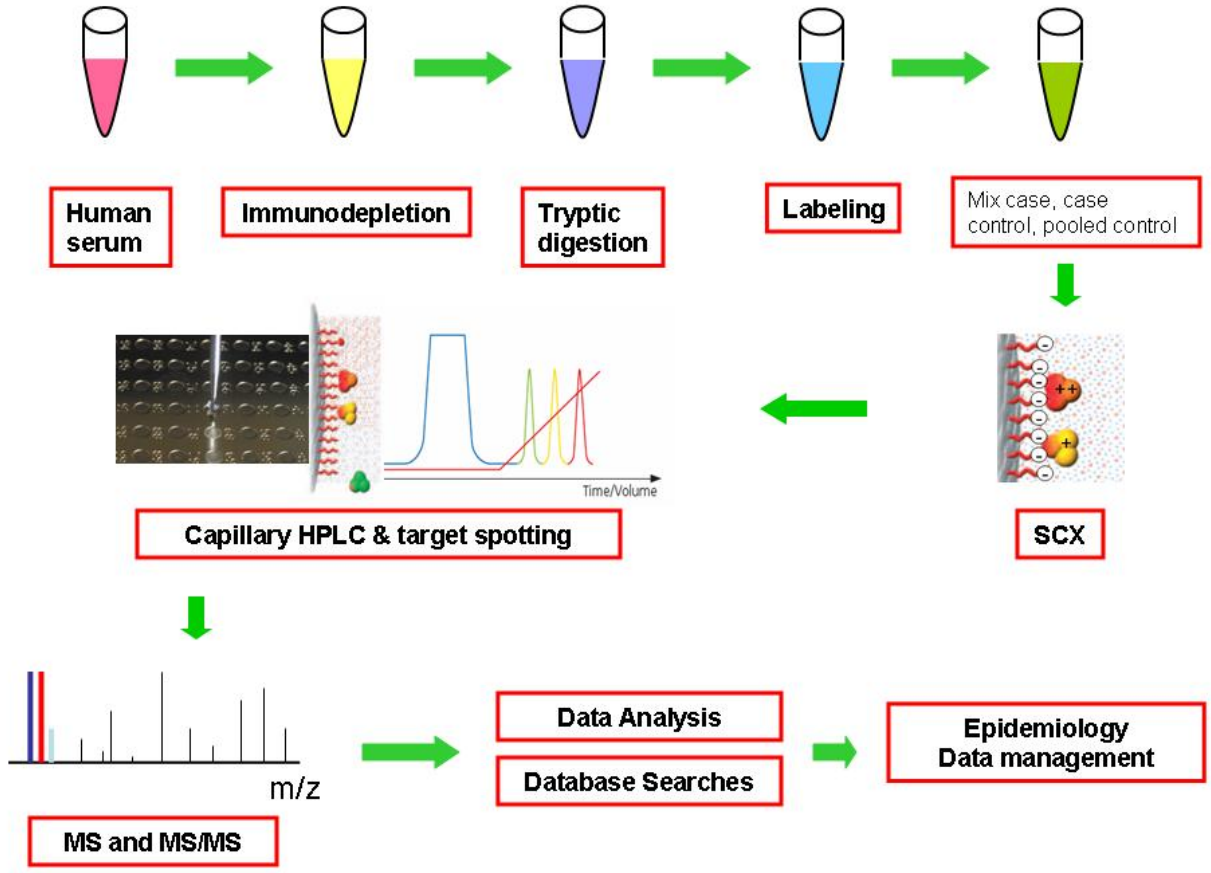


Figure 1. Steps in the proteomic analysis of breast cancer serum samples

(a) Sample preparation. The serum sample (20 µl) is loaded onto the immunodepletion column (6.4 × 63 mm, Genway Biotech Inc, San Diego, CA), the flow-through fraction is collected (Fig. 2) and its volume is reduced by ultrafiltration using a spin filtration tube with a 5 kDa Mr cutoff. The efficiency of the filtration is shown in Fig. 3. The concentrated flow-through fraction is digested overnight with 5 µg of trypsin in 20% acetrontile and 30 mM TEAB. Figure 4 shows the results of digestion performed under various conditions. 40% of the digest was subjected to labeling with one

vial of iTRAQ reagent (114, 115 or 116) overnight at 37°C. Residual reagent is quenched by adding 420 µl of water and allowing excess reagent to completely hydrolyze over an additional 30 minutes. The three differently labeled samples are subsequently mixed. To avoid any incomplete labeling of peptides, due to a possible bad batch of iTRAQ reagent, a brief test is performed by labeling a standard peptide, des-Arg-Bradykinin, prior to labeling the samples.

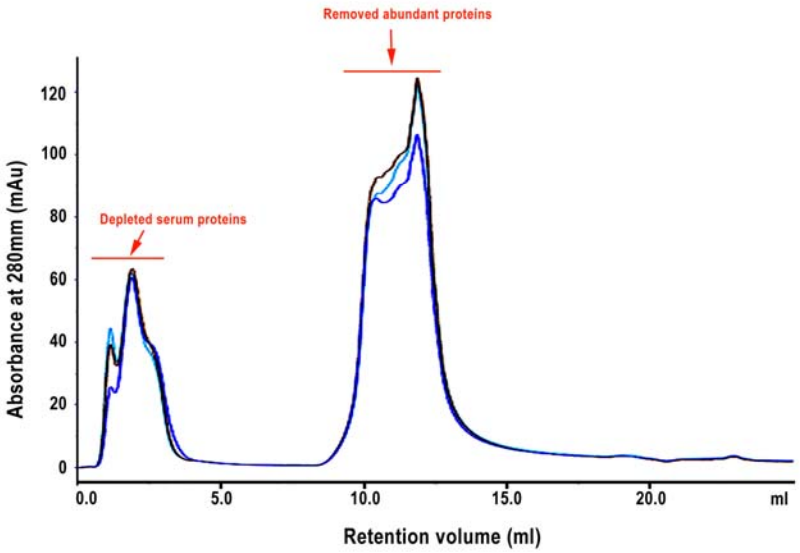


Figure 2. Immunoaffinity depletion of abundant proteins from serum. The overlay of three serum samples from one stratum is shown. The first eluting peaks are combined as the flow-through fraction for further analysis. In this combined fraction the majority (> 75%) of the 12 abundant proteins are removed.

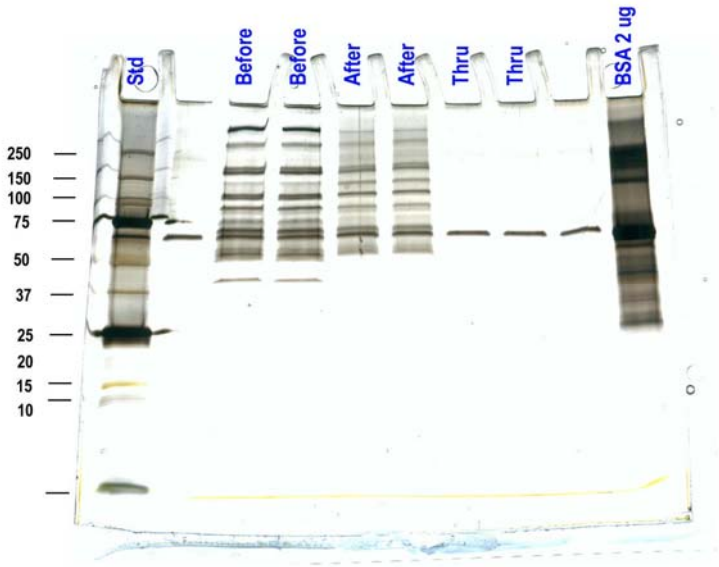


Figure 3. SDS-PAGE profile of depleted proteins before (Before) and after (After) using a filtration-concentration spin tube with a 5-kDa Mr cutoff. Equivalent amounts of samples were loaded into each lane of the gel in duplicate. The gel was silver stained revealing the resolved proteins. The spin flow-through (Thru) of the sample is also shown.

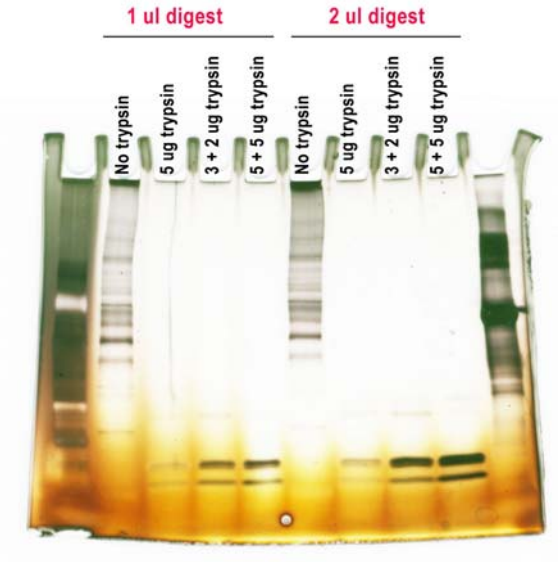


Figure 4. SDS silver stained gel of serum digested with trypsin at various amounts. One µl (left half of gel) or 2 µl (right half of gel) of digest was loaded. The digestion was done without (No trypsin), with 5 µg (5 ug trypsin), 3 µg overnight plus 2 µg for 2 additional hours (3 + 2 ug trypsin) and 5 µg overnight plus 5 µg for 2 additional hours (5 + 5 ug trypsin).

Several changes to the original procedure were necessary for optimization, including the elimination of the reduction and alkylation step of the immunodepleted flow-through fraction under denaturing conditions and its subsequent concentration on a C4 column (complete removal of guanidium hydrochloride was difficult and adversely affected the C4 column step). The end result was to streamline the procedure. The optimization of the biochemical steps was completed using standard proteins and the NIST serum standard, often used for proteomics methods development. The reduction in the amount of sample that is labeled, significantly improved labeling efficiency (to > 95%). Only 40% of the sample is now labeled with the remaining 60% being saved. A 40% proportion, corresponding to 33% of the total, proceeds to the 2D LC step. A proportion corresponding to 7% is saved for glycoprotein biomarker analysis using multi-lectin affinity chromatography; the obtained glycopeptides will be treated with PNGase-F to identify the peptides that possessed the N-linked sugars (Yang Z, Harris LE, Palmer-Toy DE, Hancock WS. Multilectin affinity chromatography for characterization of multiple glycoprotein biomarker candidates in serum from breast cancer patients. Clin Chem. 2006;52:1897-905. Plavina T, Wakshull E, Hancock WS, Hincapie M. Combination of abundant protein depletion and multi-lectin affinity chromatography (M-LAC) for plasma protein biomarker discovery. J Proteome Res. 2007;6:662-71). This glycopeptide analysis was not part of the original proposal, but may provide additional useful information. As can be seen in the detailed protocol (Appendix 1), other modifications to the sample preparation protocol were also made.

(b) Off-line 2D LC coupled with MALDI-TOF/TOF analyses. In the first dimension, the combined peptide mixture is separated by strong cation exchange (SCX) chromatography on an AKTA Purifier 10 system (GE Healthcare Bio-Sciences, Piscataway, NJ) using a PolySulfoethyl A ™ column (2.1 × 100 mm, 5 µm, 300Å; Poly LC Inc, Columbia, MD). The sample is diluted in 4.3 ml of SCX loading buffer (20% acetonitrile, 10 mM potassium phosphate, pH 3) and loaded onto the column. The column is then washed isocratically for 20 min at 0.1 ml/min to remove excess reagent. Peptides are eluted with a multistep gradient of 0-700 mM KCl (in 20% acetonitrile, 10 mM potassium phosphate, pH 3) over 15 minutes at a flow rate of 0.1 ml/minute, with fractions collected at 1-minute intervals (Fig. 5). The second dimension of the peptide separation was

performed on an Ultimate™ 3000 chromatography system equipped with a Bai Probot MALDI spotting device (Dionex, Sunnyvale, CA). Six individual SCX fraction pools were injected and captured onto a trap column (1× 15 mm; Dionex, Sunnyvale, CA) and then eluted onto an RP –C_{18} capillary column (300 µm ×150 mm; Dionex, Sunnyvale, CA) with a gradient of buffer B (buffer A, 0.1% TFA, 5% acetronitrile, 95% H_2O; buffer B, 0.1% TFA, 95% acetonitrile, 5% H_2O). Column effluent was mixed automatically in a 1:1 ratio with premade MALDI matrix, 6.2 mg/ml of alpha-cyano-4-hydroxycinnamic acid in 36% methanol, 56% acetonitrile, and 8% H2O (Agilent, New Castle, DE) using a probot MALDI spotting device and spotting directly onto MALDI plates. MALDI plates were analyzed on an AB 4700 Proteomic Analyzer from Applied Biosystems (Framingham, MA).

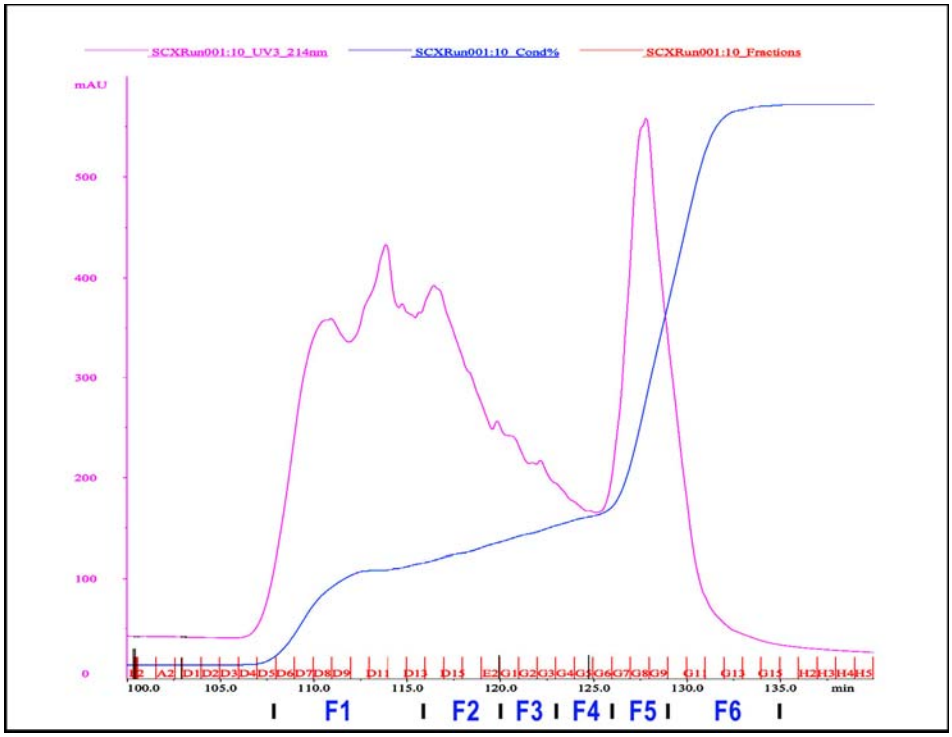


Figure 5. SCX chromatogram of iTRAQ-labeled digest of Stratum 01, showing absorbance at 214 nm (purple) and the KCl gradient (blue, 0 - 700mM) and the 6 fraction pools subjected to capillary LC and spotting onto 6 TOF/TOF target plates.

In the development of this step, the slope of the gradient was optimized so that an approximate equivalent amount of peptide ions would be detected in each of the six fraction pools (Fig. 5). This process entailed testing of various gradients by capillary HPLC and spotting the eluant onto TOF/TOF target plates, followed by data acquisition and comparison of the data sets. Each SCX fraction is deposited onto one target of 192 fractions. Optimization of the SCX chromatography condition was followed by optimization of the capillary HPLC gradient to distribute the peptides as evenly as possible across the 6 TOF/TOF target plates.

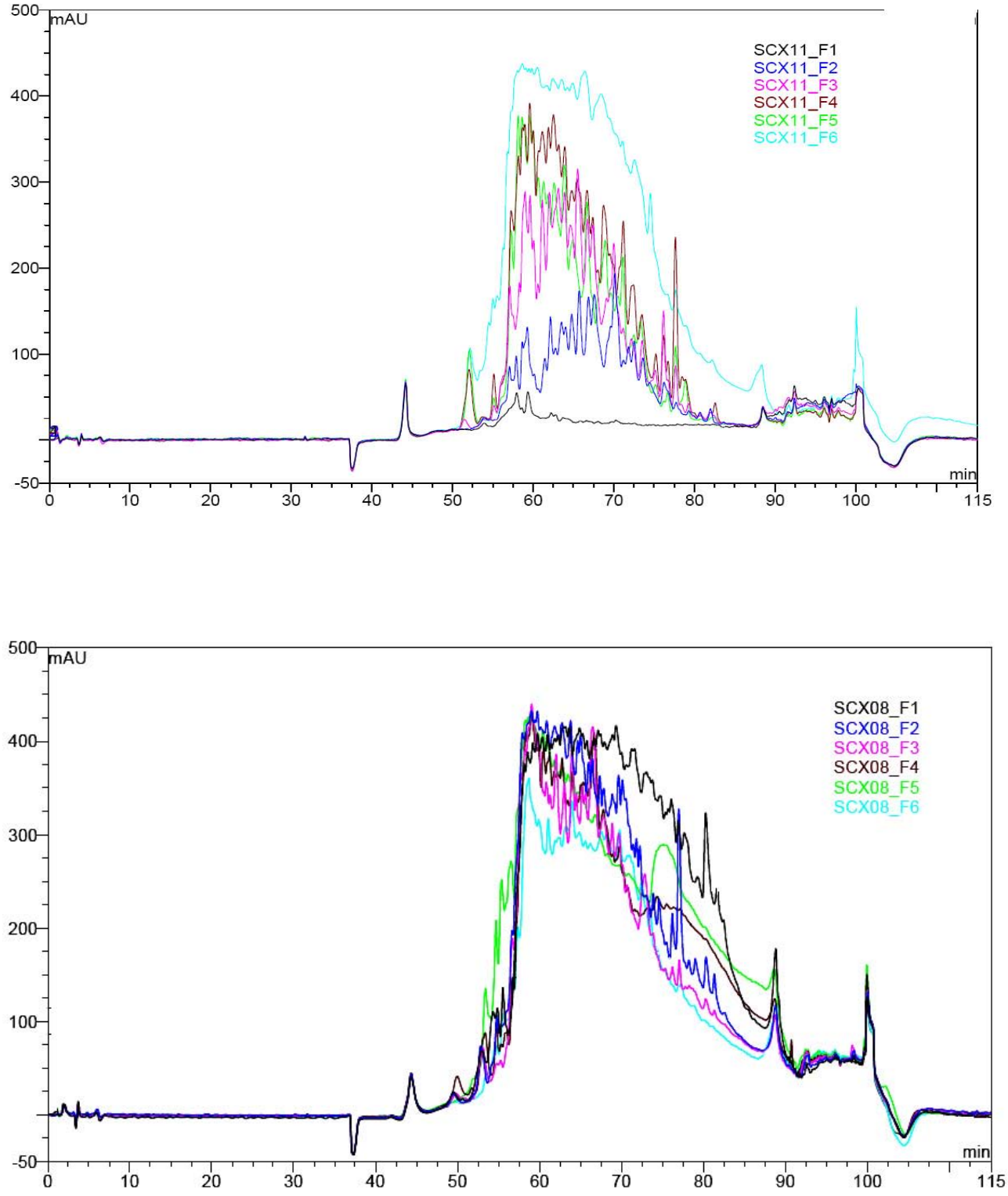


Figure 6. Overlay of the 6 LC capillary runs of each of the pooled SCX fractions. Improvement of peptide distribution among the 6 fractions is shown. In the top panel the uneven distribution of peptide amounts from each of the 6 SCX fraction pools is clearly shown. The lower panel displays the uniform amounts and distribution of peptides from each of the 6 SCX fraction pools after

optimization. Fractions eluted between 50 - 90 min from each LC run were spotted onto a single 192-well MALDI target plate (therefore, there are 6 plates per stratum).

(c) Data processing. Data analysis was performed using in-house software (Du P, Angeletti RH. Automatic deconvolution of isotope-resolved mass spectra using variable selection and quantized peptide mass distribution. Anal Chem., 2006, 78:3385-92, 2006, P Du, R Sudha, MB Prystowsky, R Hogue Angeletti; Data reduction of isotope-resolved LC-MS spectra. Bioinformatics, 2007, Jun 1;23(11):1394-400) to build arrays with SCX fraction number, retention time, precursor monoisotopic mass of all ions containing the iTRAQ reporter ions (114, 115 and 116) along with their signal intensities. The retention time drift is corrected for by using the retention time of the internal standard peptides. Peptides having similar mass (± 0.1 Da) and retention time (± 60 sec) were considered to be the same peptide. Intensity levels of ions at m/z 117 were used as indicators of noise level. In analyses of replicate samples of the pool, a high level of reproducibility was observed for all iTRAQ reporter ions having intensities greater than 500 counts (Fig. 7).

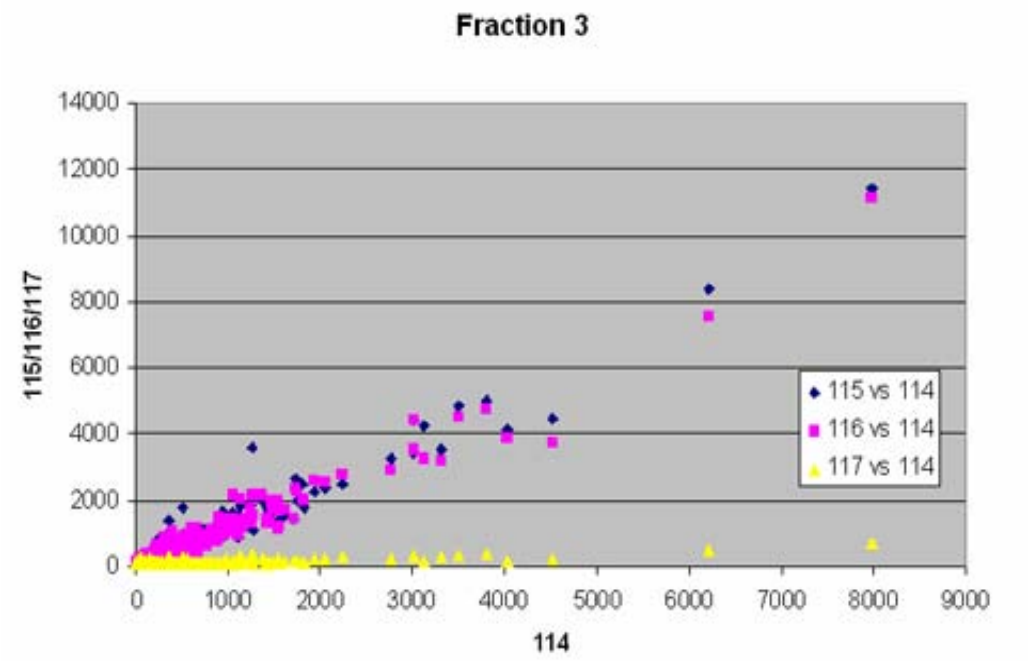


Figure 7. Plot of the iTRAQ reporter ion intensities (*m/z* 114, 115 & 116) of replicate pooled serum samples. The ion intensity value plot of *m/z* 117 depicts our baseline noise level. Ideally, the ion intensity values from *m/z* 114, 115 & 116 should all be equal to each other. The calculated correlation coefficients are: 114:115, 0.9701; 114:116, 0.9719; and 115:116, 0.9811.

In addition, the data are searched through the TOF/TOF GPS server and Mascot to identify the sequenced peptides and their parent proteins. As an example, from the stratum 02 immunodepleted serum, more than 1,098 proteins can be identified with a Confidence Interval (C.I.%) of 95% or greater. It should be noted that in the quantitative analysis of serum, most peptides will be unchanged. In the present experiments, this will mean that in most cases the 114 and 115 marker ions (representing blinded case and case control samples) should be indistinguishable from the 116 marker ion, the pooled control (Figs. 8 and 9). Of the 1,098 proteins identified, 305 proteins are highly relevant to pathways and disease processes and are not in the upper tier of abundant proteins (Appendix 3).

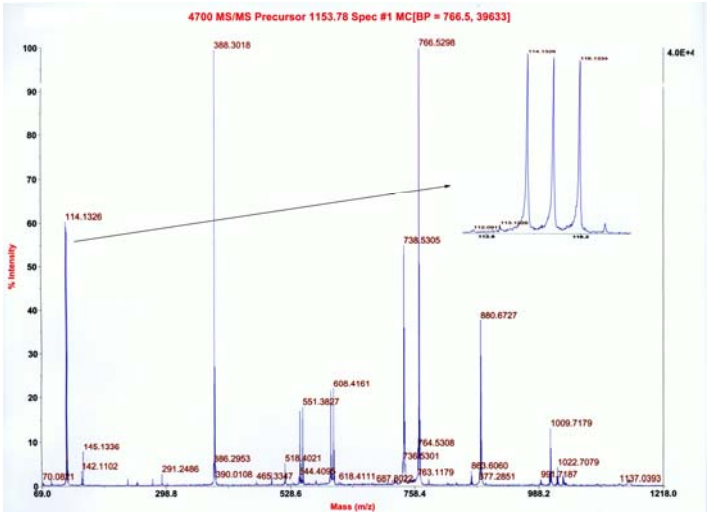


Figure 8. MS/MS spectrum of a peptide ion with approximately equal intensities of the iTRAQ reporter marker ions for case, control, and pooled control. The inset is a magnification of these marker ions (*m/z* 114, 115 & 116).

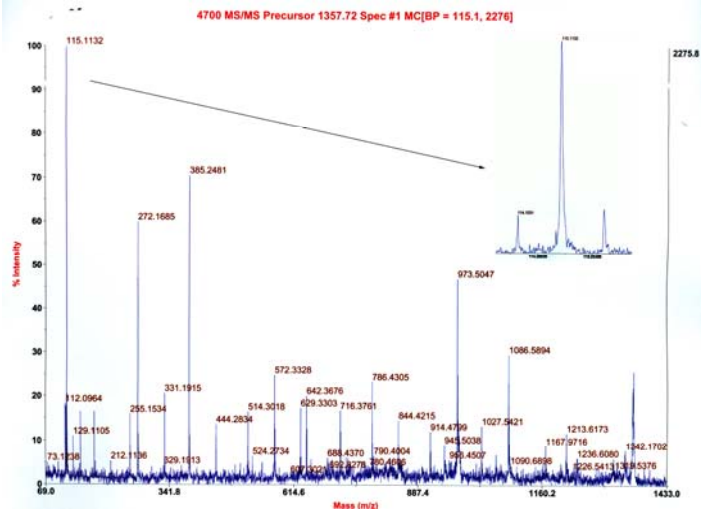


Figure 9. MS/MS spectrum of a peptide ion with an intense 115 iTRAQ reporter marker ion (inset), indicating the increased expression of this peptide in either the case or control serum.

Using the Protein Interrogation of Gene Ontology and KEGG databases web-based program (PIGOK; http://128.40.158.133/pigok.html) the proteins identified from part of stratum07 (Fractions 4 and 5, 500 hit search, minimum modifications) were categorized and are shown below in figures 10 to 12.

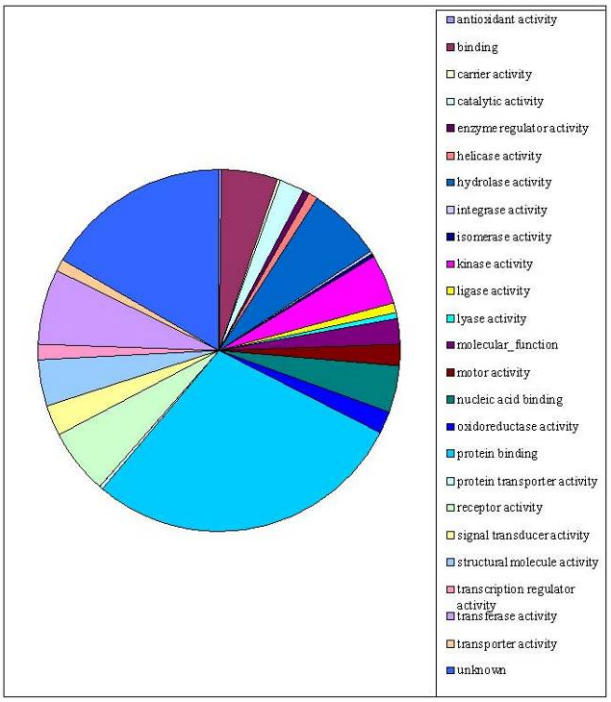


Figure 10. Categorization of proteins according to their molecular functions using PIGOK. These proteins are identified using the GPS Explorer program. MSMS data were taken from SCX fractions 4 and 5 of stratum7.

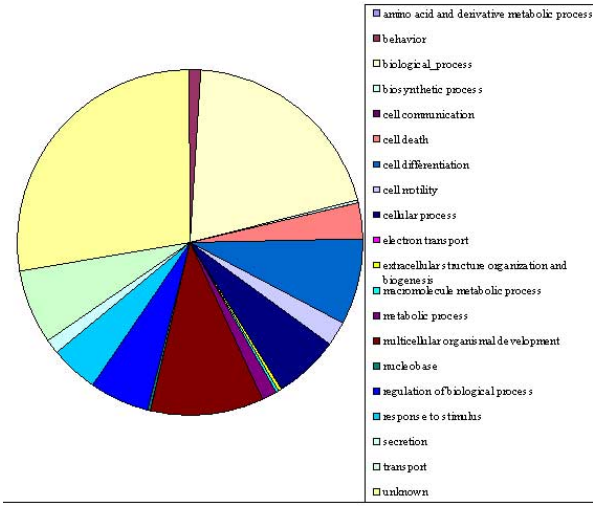


Figure 11. Categorization of proteins according to their biological process using PIGOK. These proteins are identified using the GPS Explorer program. MSMS data were taken from SCX fractions 4 and 5 of stratum7.

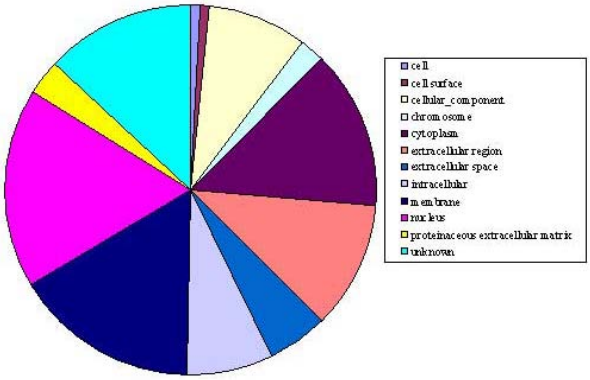


Figure 12. Categorization of proteins according to their cellular_component using PIGOK. These proteins are identified using the GPS Explorer program. MSMS data were taken from SCX fractions 4 and 5 of stratum7.

It is notable that low abundance proteins are being consistently identified in these experiments. For example, proteins such as NF-IL6 and interferon $\alpha 2/\beta 1$ are reported to be present at levels less than 200 pg/ml, whereas serum albumin is present at approximately 35-50 mg/ml. This would represent a dynamic range of detection of almost 10^8 orders of magnitude.

vi) Data and operations management

The process of tracking samples and data throughout the complicated process from the initial immunodepletion phase through the MSMS phase is crucial in this study. Each of the procedures in this process has been documented in an operations manual and checklists have been devised for each step of the process (Appendices 1 and 2) to ensure that all procedures are in place and are adhered to strictly. In order to track the many different test tubes that are produced at each juncture, cryo-labels have been created that clearly identify the current procedure and the sample ID number.

Data are stored on three different computers, each of which is connected to the equipment necessary for each step of the process. Directories have been created in each of these machines to clearly identify project data and these directories are backed up weekly onto a portable hard drive. After analysis of each case-control stratum is completed, the hard drive is copied to the data management servers and data on the hard drive are deleted so that data resulting from the analysis of the next stratum can be backed up.

During the MSMS phase, data files for each of the six fractions are created. These files are then converted into separate Td2 files in order to prepare analysis files. An analysis program is run on each of these files and space delimited text files are outputted which contain one line of data for each spectrum in the given fraction. Data included in this file include the spot number (spectrum), M/Z, mascot score, protein accession number, and the intensity for each sample by peptide. Quality assurance procedures include steps to ensure that the file is created correctly before raw data files are moved. After these interim analysis files are created, all data for each stratum are merged into one file which will be used to create the final analysis file for the study.

Project management is facilitated by a portal web site which has been created for this study and allows all members of the study to collaborate in a shared environment. The portal site hosts a project calendar and a tracking system which allows each member to update the status of each sample as it is completed and document issues that occur. The data manager is automatically notified when this system is updated to ensure that the project is tracked efficiently and monitored appropriately.

vii) Key research accomplishments We have developed an integrated, highly sensitive, proteomic analysis involving fractionation by immunodepletion and multi-dimensional HPLC, analysis by MALDI-TOF/TOF-MS and sophisticated computational analysis of the data, to tackle the complexity and dynamic range of the serum proteome. We have shown that this procedure identifies proteins that are highly relevant to pathways and disease processes and that are not in the upper tier of abundant proteins. We have established a sophisticated procedure for operations management and quality control.

viii) Reportable Outcomes So far our publications have been on the development of algorithms for signal processing:
1. Du P, Angeletti RH. Automatic deconvolution of isotope-resolved mass spectra using variable selection and quantized peptide mass distribution. Anal Chem., 78:3385-92, 2006,
2. P Du, R Sudha, MB Prystowsky, R Hogue Angeletti (2007) Data reduction of isotope resolved LC-MS spectra. Bioinformatics, in press)

ix) Conclusions Using the final procedure outlined herein, we have completed the MS analysis of the first 9 strata. A reproducibility study is currently ongoing on 6 of the pooled samples, with 3 in each of 2 runs, to mimic 2 strata (i.e., 3 pooled samples in one stratum and then 3 pooled samples in another). This will yield measures of intra- and inter-run repeatability.

Our motivation for intense data acquisition to complete this project has revealed shortcomings of the mass spectrometer (Applied Biosystems 4700 Proteomics Analyzer) used for data collection, namely its inability to operate full-time due to charging effects now known to affect instrument performance. This has limited the number of plates that can be used per day and per week before either needing to allow the instrument to discharge or having to break the vacuum and clean. Because of these problems, which have delayed the project, we are engaged in attempts to trade-in the 4700 instrument for the 4800 model, which we have determined is able to operate full-time, but will still requiring regular cleaning. If we are able to upgrade the instrument, the project would be accelerated.

While we are behind schedule, we believe that we have developed procedures that provide the complexity required to uncover new markers of disease. Furthermore, we have initiated the analysis of the actual study specimens at a reasonable rate. The personnel involved are highly trained and familiar with all aspects of the project. We are now in the intense data acquisition phase and all personnel are highly motivated to complete the project. Indeed, if necessary, it is our goal to seek funds from other sources to accomplish this.

Due to the fact that we are behind schedule, we have decided to adopt two strategies that will reduce the time required to complete the project:

Firstly, we will reduce the sample size for the training phase of the study to 32 case-control pairs. A sample size of 32 matched pairs will still offer over 90% power to detect the 70% sensitivity/95% specificity specified in the original grant, corresponding to a conditional odds ratio of over 11, and over 80% power to detect a conditional odds ratio of 5, which would result from 63% sensitivity and 88% specificity. Thus, with the proposed reduction in sample size, the study will still provide adequate statistical power to detect reasonably large differences between cases and controls. Reducing the size of the training set to 32 case-control pairs would save approximately 2 months.

Secondly, we have decided to abandon the development of immunoassays. We propose instead to analyze the test set of 20 case-control pairs using the same protocol used to analyze the training set. This would not compromise our ability to test the proteomic patterns identified in the training phase to discriminate between cases and controls in the test phase. Once the marker proteins are known, the development of the immunoassays can be performed at a later date. We estimate that this will save us at least 6 months.

Appendix 1

Proteomic Lab Operation Manual

Table of contents

I. Test tube preparation

A. For the first phase of this study, we will be analyzing 40 case control pairs. Each case control pair has been assigned a stratum number (01-40). Each sample has a unique ID number (1-80). The lab will receive 5 aliquots for each one of the samples. For each case-control pair (i.e. stratum), the lab will also receive a pooled sample. The labeling schema developed for this study identifies each test tube as: STRATUM# _ ID # _ ALIQUOT #

Example: For the first stratum (01), the lab will receive 3 test tubes.

- The first test tube will be labeled 01_1_1. 01 represents the stratum number. The 1 in the middle represents the ID for this sample. The last 1 represents the 1st aliquot for this sample.
- The second test tube will be labeled 01_2_1. 01 represents the stratum number. The 2 represents the ID for this sample. The last 1 represents the 1st aliquot for this sample.
- The third test tube will be the pooled sample for the 01 stratum and will be labeled 01_PL_1. 01 represents the stratum number. The PL represents POOL for this sample. The last 1 represents the 1st aliquot for this pooled sample.

B. A label sheet will be prepared by the Department of Epidemiology and Population Health (DEPH) for each stratum. The sheet will contain all of the labels that are needed for the process. Each label contains the procedure step name (CONC, DIGEST, iTRAQ etc) and the numbers used to identify the sample. The first 2 columns of the label sheet contain the labels for the first aliquot of each stratum. The other 4 columns contain extra

labels for the other aliquots in case they are needed. As a rule, only the first 2 columns of the label sheet will be used unless a 2nd aliquot is needed for a particular sample.

II. Immunodepletion
(Removal of major abundant serum proteins by immunodepletion chromatography, 3 serum samples consecutively at a time)

Buffers

Buffer A1: Phosphate Buffered Saline (PBS), pH 7.4, from Sigma (P3813-10PAK, contains 0.01 M phosphate, 0.138 M NaCl, 0.0027M KCl). Freshly prepare before use for the day.

Buffer B1: Stripping Buffer: 0.1 M glycine-HCl, pH 2.5, containing 0.02% sodium azide.

Neutralization Buffer A2: 0.1M Na_2HPO_4-NaH_2PO_4, pH 8.0, containing 0.02% sodium azide.

Column Storage Buffer: PBS containing 0.02% sodium azide.

Filter all buffers through a 0.2 µm sterile membrane filter and store at 4°C, except buffer A1 which need to be prepared freshly each time.

Column

6.4 × 63 mm, affinity column from Genway Biotech Inc (San Diego, CA) Cat. Number : MIXED12-LC12. Now it is sold as 'ProteomeLab-IgY column kits' by Beckman. **The column must be stored at 4°C in column storage buffer. The column must be washed with 3 column volumes of room temperature degassed dilution buffer IMMEDIATELY after it is taken from 4°C storage**. This procedure will prevent air bubble formation inside the column.

Instrumentation

AKTA purifier
Injection loop size: 250 µl
Flow cell (Cat. 18-1147-25): 3 mm pathlength, 0.7 µl hold up volume.
(Check the synchronization of UV tracing and sample collection of the fraction collector)
Syringe: 250 µl syringe from Hamilton

Procedure

1. Bring PBS, Stripping buffer, neutralization buffer and column storage buffer to room temperature and re-dissolve precipitates. (Phosphate precipitates when stored at 4°C). Degas all buffers by purging the buffers with helium for 10 minutes.
2. Turn on AKTA work-station and computer. Wait for system synchronization to complete.
3. Connect the 250 µl sample loop to the correct valve inlet position.
4. Connect UV light, pH meter, and Conductivity meter to the correct position.
5. Run the *equilibrate-system* program under Method, to bring all solutions to running-condition.
6. Switch valve to "inject" position to wash and purge the loop with 2 ml of Buffer A1. Then switch valve to "load" position to wash and purge the loading syringe adaptor and the loop with 2 ml of Buffer A1 from a syringe fitted with the loading needle.
7. Connect the depletion column immediately after brought out from 4°C, and purge with 10 ml of Buffer A1 at 1ml/min. Make sure that no air is introduced to the column.

8. Test the fraction collector tubing to make sure it's not clogged by switching the Frac to F2 and flow for 5 ml through the collector outlet.
9. Dilute 20 µl serum sample with 80 µl PBS and spin at 13,000 rpm for 5 min to pellet insoluble debris.
10. End any run program.
11. Insert 10 1.5 ml microfuge tubes to position A1 to A10 on the 12mm collector plate.
12. Draw up the 100 µl supernatant with 100 µl syringe and inject the sample into the loop. Do not remove the syringe from the injection port until the current run is finished.
13. Using the *Rohan\DepletionRunMethod* program (Appendix 1)under depletion method saved on the AKTA purifier, start the run.
14. After finishing all runs, wash the immunodepletion column with 3 column volumes of column storage buffer and store the column at 4°C.
15. Pool the flow-through fraction A3 to A8, with absorption at 280 nm into a 15 ml round-bottomed polyethylene tube (with cap), about 2.5 ml. Place the appropriate CONC label on the tube. Put on ice.
16. Save the 3 depletion files for this triplet (case, control and pool) in the Rohan directory. The naming convention for these files should be:
    a. 'DP'<Stratum#><ID#><Aliquot#>
       Example: DP010101
                DP010201
                DP01PL01
17. When the process is finished, back up the 3 deletion files onto the Rohan portable hard drive in the DEPLETION directory.
18. Integrate the depletion curve to calculate the peak areas. Use this as quality control within and among stratum. Print out the integrated curves and the integrate results./

III. Trypsin Digestion
(concentration, buffer exchange and trypsin digestion; process & digest the 3 samples from section 1 above simultanously)

Buffers & reagents

5% Triton X-100 containing 0.02% sodium azide, store at room temperature.
50 mM TEAB (triethylammonium bicarbonate), freshly prepared from 1M stock.
TPCK treated trypsin, 20 µg /vial (Promega, Cat. # : V5111), store at -20°C.

Procedure

19. Pre-treat the 5 kDa cut-off Ultra-4 centrifugal concentrator (Millipore, Bedford, MA 01730. Size: 5,000 MWCO. Cat. No. UFC800596, 96pk) with 4 ml 5% Triton X-100 for 30 min at room temperature, followed by extensive washing with distilled water to remove the Triton X-100.
20. Transfer the pooled fractions from step 15 to the concentrator prepared in step 19.
21. Spin at 3,960 x g for 45 min at 8°C using a swinging bucket centrifuge to reduce the volume to 80 µl or less. This can be judged against the calibration line on the tube.
22. Add 3 ml of 50 mM TEAB (triethylammonium bicarbonate buffer, Fluka, lot and filling code: 1192744 34005180) in the concentrator to change buffer to trypsin digestion solution (50 mM TEAB).
23. Concentrate as in step 21 to 80 µl or less.

24. Add another 3 ml of 50 mM TEAB and spin as in step 21 to reduce volume to 80 µl or less.
25. Transfer the concentrated samples to a another set of 500ul tubes (label the tubes first) separately.
26. Set the 100 µl pippet at 80 µl. Draw up the sample to the tip to the maxium of the sample volume, compensate the difference with 50 mM TEAB to 80 µl. Add 20 µl acetonitrile to each tube thus to make the final sample containing 20% acetonitrile.
27. Dissolve 1 vial of trypsin in 20 µl of 50 mM TEAB. Each vial contains 20 ug TPCK treated trypsin (Promega, Cat: V5111).
28. Add 5 µl of trypsin preparation to the sample at step 26. The total digestion volume is 105 µl.
29. Incubate at 37°C overnight at 400 rpm on a thermomixer.

IV. iTRAQ labeling
(Label the 3 samples from section 2 above simultaneously, each with a unique iTRAQ reagents)

Buffers & reagents:

100% ethanol as supplied in the iTRAQ kit
iTRAQ reagents 114, 115, 116 from the "iTRAQ Reagent Multi-Plex Kit" from Applied Biosystem. Store at -80°C.

Procedure

30. Spin down the digests resulted from step 29
31. Move one set of iTRAQ reagents and ethanol to room temperature, wait for 20 min. Spin the reagents briefly.
32. Add 105 ul 100% (absolute, HPLC grade, from iTRAQ kit) ethanol to each vial of iTRAQ reagent. Votex to dissolve the reagent completely. Spin briefly to collect all solution to the bottom.
33. Test the quality of the iTRAQ reagents by adding 3 ul of each labeling reagent to 1 µl of the 1:10 (in 50 mM TEAB) diluted 2 mix peptides (bradykinin-904 Da and ACTH-2465 Da), for 45 min at room temperature.
34. After labeling, add 6 ul of H2O to each tube, mix. Take 3 µl of each into one 500 µl tube, add 9 µl of the matrix, Mix.
35. Detect the MS and MSMS profile of the bradykinin using the 4700 instrument. If (1) in MS mode no 904 is observed while 1048 appears instead, and (2) in MSMS mode of 1048 as parent ion, the product ions 114, 115, and 116 are labeled equally, continue the labeling of serum sample with the rest of the reagents as following.
36. Transfer 40 µl of each digest to each corresponding iTRAQ reagent vial, vortex each tube to mix. Spin briefly. Incubate the labeling at room temperature overnight.
37. Save the rest 60% of digest at -80C.
38. Stop the labeling by adding 420 µl of SCXA1 and allow excess reagent to completely hydrolyze over an additional 30 min at room temperature. Spin down briefly.
39. Combine 466 µl (out of 560) of each to a 50 ml tube. Add 4,300 ul SCX (strong cation exchange) buffer A. Save the rest 94 (1/6 of the labeled sample) at -80C.
40. Place the iTRAQ label for the current stratum on the tube.
41. The combined and diluted sample (5,698 µl) should be at pH 3.0 and can be stored at 4°C overnight. (It will contain 0.7 mM TEAB, 2.2% ethanol, 9.15 mM potassium phosphate,

18.3% acetonitrile. The total concentration of salt and organic solvent are close to SCX buffer A1)

V. SCX-strong cation exchange chromatography

Column

PolySulfoethyl A column (2.1x 100 mm, 5 µm, 300Å, volume≈0.3ml) from Poly LC Inc. Cat. No. 102 se0503
Guard column for the above

Buffers:

Buffer A: 25% acetontrile, 10 mM potassium phosphate, pH 3 (Made by titrating phosphoric acid with 5M KOH to pH 3.0)
Buffer B: 25% acetontrile, 10 mM potassium phosphate, pH 3, 700 mM KCl.

Instrumentation

AKTA purifier
Injection loop (8 ml)
Flow cell (Cat. 18-1147-25) (3 mm path length, 0.7 µl hold up volume)
(check the synchronization of UV tracing and sample collection of the fraction collector).
Syringe: syringe from Hamilton (10 ml), cat. No.: 7650-01; or 10 ml PP/PE lubricants free syringe (10 ml).

Run Method

See Appendix 2.

Procedure

42. Check that all solutions (A1 and B1) are ready. Check if the pH is correct, otherwise adjust it to pH 3.0. Filter if needed.
43. Turn on AKTA work-station and computer. Wait for system synchronization to complete.
44. Connect the 8 ml loop to the right position.
45. Connect UV light, pH meter, and Conductivity meter to the right position.
46. Run the *SCX equilibrate-system* program under Method, to bring all solutions to running-condition.
47. Switch valve to “inject” position to wash and purge the loop with 20 ml of Buffer A. Then switch valve to “load” position to wash and purge the loading syringe adaptor and the loop with 20 ml of Buffer A from a syringe fitted with the loading needle.
48. Connect the PolySulfoethyl A column (connect the guard column first) to the right position of the flow path.
49. Wash the column with 2 ml of buffer A at 0.1 ml/min.
50. Test the fraction collector tubing to make sure it’s not clogged.
51. End any run program.
52. Insert 1.5 ml tubes to collector rows A1 to A3, D1 to D16, E1 to E2, G1 to G16 and H1 to H10.
53. Draw up the sample from 41 with syringe and load the sample into the loop. Do not remove the syringe from the injection port until the current run is finished.
54. Using the *Rohan\SCXRunMethod* method saved on the AKTA purifier, start the run. Collect the eluted peptide. Combine fractions D8 to D13 as ***F1***, D14 to E2 as ***F2***, G1 to G3 as ***F3***, G4 to G7 as ***F4***, G8 to G11 as ***F5***, G12 to H3 as ***F6***. There might be slight variation in

combination method, depending on the SCX spectrum profile. Compare with previous SCX runs to decided the exact combination method. Store at 4C if not using immediately.

55. After the run, regenerate the column by running the column with a blank gradient followed by 6 ml of buffer A. Finally, wash the column and the system with at least 20 ml of 20% ethanol and store the column in 20% ethanol at room temperature.
56. Save the computer file generated by this process in the Rohan directory and on the portable hard drive in the iTRAC subdirectory. The naming convention for these files should be:
    b. 'iTRAQ'<Stratum#><Aliquot#>
        Example: iTRAQ0101
57. Integrate the curve to calculate the elute peak areas. Set the X-axis window as 10 to 14 ml, Y-axis window as 0 to 1200 mAu at UV214. Re-set the baseline by drawing points. Print out the integrate results.

VI. RPLC-reversed phase chromatography (Interfaced with Probot automatic spotting)

Columns:

Trap column
    Dionex P/N 6720.0012
Loop
    250 µl
RP C18 column
    Dionex, P/N 160295, 300 um I.D.x, 15cm, static phase: C18, PepMap100, 3um, 100A
ABI 4700 Analyzer compatible disposable 192 well MALDI insert plate

Solutions:

Solvent A
    5% acetronitrile, 0.1% TFA
Solvent B
    95% acetontrile, 0.1% TFA

Run Method

See Appendix 3.

Procedure

58. Speed-Vac each SCX fraction to 98 ul. Adjust to 0.1% TFA by adding 1 ul of 10% TFA. Add 1 ul of LC internal calibration peptides.
59. Label 6 U3000 LC sampling vials as F1. F2, F3, F4, F5 and F6.
60. Transfer the Fractions to each designated LC vial.
61. Take 6 new disposable plate insterts, assign the SCX fraction to a plate by writing dwon the plate number..
62. Spot the standard calibration peptides in the 6 calibration spots of each plates.
63. Put the plates on the magnetic insert holders and place them in order on the Probot platform
64. Set the Dionex ultimate 3000 and the Probot at working condition
65. Place 6 disposable 192-well MALDI plate pre-spotted with calibration standards on the Probot.
66. Put the LC sample vials in Autosampler positions RA1 to RA6
67. Start the Sequence program SCX_0X for LC and spotting, each run takes 115 min.

68. When each step is complete, save the computer files generated by this process in the Rohan directory and a second copy on the portable hard drive in the SCX directory. The naming convention for these files should be:

c. 'SCX'_<Stratum#>_<Aliquot#>_'FR'<Fraction#>

Example: SCX_01_1_F1
SCX_01_1_F2
.
.
SCX_01_1_F

69. After all runs, regenerate the column by running the column with a blank gradient followed by 1 ml of buffer A. Finally, wash the column and the system thoroughly with 70% isopropanol and store the column in 70% isopropanol at room temperature.

VII. MS/MS
(ABI 4700 MALDI-TOF/TOF for MS and MS/MS of iTRAQ labeled peptides)

70. On the MSMS computer, create a sub-directory in the Rohan directory for the stratum about to be processed. The naming convention for this sub-directory should be:

d. 'MS'_<Stratum#>_<Aliquot#>

Example: MS_01_1
MS_02_1
.
.
MS_40_1

71. Begin the MSMS program. The computer will create a sub-directory for each plate (fraction). The naming convention for these directories should be:

- 'MS_S'<Stratum #>_'F'<Fraction #>

Example: MS_S01_F1
MS_S01_F2
.
.
MS_S01_F6

72. Open the ABI4700 software

73. Load the plates in the autoloader, record the slot number, one plate each time.

74. Create new spot set for each plate (xxxx_BCPP_Stratumxx_MALDI_Fx,…) using the correct LC/MALDI spot set template

75. For MS calibration, in the Spot Set Manager, select the Calibration spots for Job-Using Run_Specific Methods, choose the Acq Method for MS calibration, choose the corresponding Proc Method.

76. For MS/MS calibration, in the Spot Set Manager, select the Calibration spots for Job-Using Run_Specific Methods, choose the MS/MS Acq Method for MS/MS at 1570.60 m/z, choose the corresponding Proc Method for 1570.60 m/z.

77. For sample run, in the Spot Set Manager, select the sample spot rows from Job-Using Run _Specific Methods, choose the Acq Method form MS service runs, choose the Proc Method of internal calibration (903 and 2465 m/z).

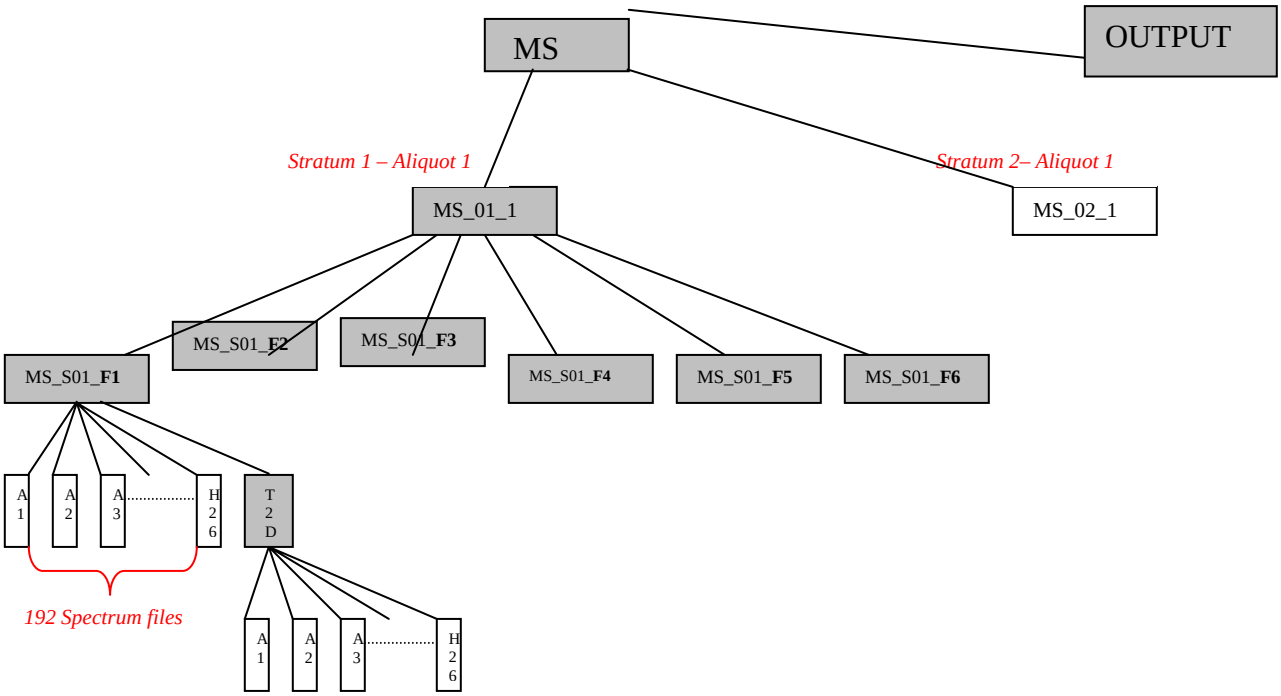
78. Select the Run job-wide interpretation function. In Cal Types Updates, select None. In the interpretation method, choose the correct methods for MS/MS acquisition and processing (these methods need to be tested and updated every before every batch run). Select Chromatogram Peak Width Number as 2. Parent ion selection from 900 to 3000 m/z.
79. For the first 3 fractions, set up all spots as one job run.
80. For the rest of the fractions, set up spots A to B, C to D, E to F, G to H as each single job run. Use Negative scans to flush the ion dusts for 10 seconds, then 10 min and then 10 min again before each positive scan.
81. Save each spot set.
82. Map spot set to the corresponding plate number and slot number
83. Load each plate to align the plate position
84. Test the acquisition method, processing method, and interpretation method with the calibration standard peptide
85. Calibrate for mass accuracy.
86. Re-select the newly updated methods for each spot set.
87. Save the spot set again.
88. Submit the spot set job to queue run, observe the validation process
89. Switch the acquisition mode from interactive to batch mode
90. Start the queue run
91. Observe the beginning of the automatic run, pay attention to the signals.
92. After each fraction run, draw the 116 signal dot-plot to see the distribution of 116 reporter against retention time. If the distribution doesn't match the LC profile, the affected spots need to be re-run.

VIII. Data analysis (Protein ID and label ions comparison)

In this step, the spectrum will be analyzed and the volume of the ion peaks of the labels will be compared. At the conclusion of the MSMS run for each stratum, the following procedures will be run.

Procedure

93. Connect the portable hard drive to the MSMS computer, open Windows Explorer, highlight the MS directory name for the stratum(e.g. MS_01_1) and copy the entire MS directory for the current stratum into the MS directory on the portable hard drive. Open the spotset, display MS and MS/MS runs, highlight all rows from A1 to H2, right mouse click on the highlighted rows, select export to folder, select or create the application folder, export as T2D files.
94. Connect the portable hard drive to the Unix machine.
95. On the portable hard drive, create a sub-directory in each fraction directory called T2D. Convert all files in each of the 'Fraction' directories for the stratum T2D files format using the web-based program and place them in the T2D directory for that stratum. A figure representing the directory structure for each stratum appears below*: (Directories are shaded)*



96. A program will be run on all .t2d in a given fraction directory and will create a space delimited text file in the MS/Output directory on the portable hard drive. This file will contain 192 lines (one line for each spectrum in the given fraction). The name of the file will be 'S'<Stratum#>_'F'<Fraction#>pepdata.txt (e.g. S01_F1pepdata.txt for Stratum1/Fraction1) and it will contain the following data fields:

not normalized

MS2 spectrum data

Fraction #	Spot #	M/Z (peptide mass data)	Intensity for 114	Intensity for 115	Intensity for 116	AA Sequence	Mascot Score	Protein Asc #							
F1	A1														
F1	A2														
F1	A3														
F1	A4														
F1	A5														
F1	.														
F1	.														
F1	B1														
F1	B2														
F1	.														
F1	.														
F1	C1														
F1	C2														
F1	.														

F1	.														
F1	.														
F1	.														
F1	.														
F1	.														
F1	.														
.	.														
.	.														
F1	H26														

97. After the program is run, the .txt file should be opened to confirm that it was created properly.

98. Proceed to run the program on the remaining fraction directories for the given stratum.

IX. Data Management:

A. Data are backed up onto the portable hard drive after each stage of the process (see sections III.17, V.51, VI.58, VIII.64 above). After a complete run on a stratum is complete (approximately once every week to 10 days) , the DEPH will copy the hard drive onto their servers, delete all data on the hard drive and return the hard drive to the lab so that analysis on the next stratum can begin.

B. The DEPH will merge the output files for all fractions for each stratum (i.e. S01_F1pepdata.txt , S01_F2pepdata.txt etc.) into one output file for each stratum (S01final.txt, S02final.txt etc.). A program created by the proteomics lab will be run on these stratum files to create the final analysis file which will contain the following data:

Peptide Mass	Retention Time (Spot #)	Stratum 01 (114) Intensity After Normalization	Stratum 01 (115) Intensity After Normalization	Stratum 01 (116) Intensity After Normalization	Stratum 02 (114) Intensity After Normalization	Stratum 02 (115) Intensity After Normalization	Stratum 02 114 Intensity After Normalization		Stratum 40 116 Intensity After Normalization

Appendix

1. Method of Depletion Run
2. Method of SCX Run
3. Method of LC Run

1. Method of Depletion Run

Buffer A1: Phosphate Buffered Saline (PBS), pH 7.4

Buffer B1: 0.1 M glycine-HCl, pH 2.5

Buffer A2: 0.1M Na_2HPO_4-NaH_2PO_4, pH 8.0

0.00 Valve at Load position, inject the sample to loop
0.00 Equilibrate the column, 100% buffer A1, for 0.1 min, at 0.1ml/min
1.00 Valve at Inject position (loading column), 100% buffer A1, for 4 min at 0.1 ml/min
1.00 Start fraction collection at 0.5 ml/fraction, for 10 fractions
5.00 Switch Valve to Load position, 100% buffer A1, for 18 min at 0.1 ml/min
26.5 100% buffer B1, for 10 min at 0.1 ml/min
36.5 100% buffer A2, for 10 min at 0.1 ml/min
46.5 100% buffer A1, for 10 min at 0.2 ml/min
56.5 Run end.

2. Method of Strong Cation Exchange (SCX) Run

Solution A1: 10 mM potassium phosphate, pH 3.0, 20% acetonitrile
Solution B1: 10 mM potassium phosphate, pH 3.0, 700 mM potassium chloride, 20% acetonitrile

0.00 Valve at Load position, inject the sample to loop
0.00 Equilibration the column, 100% Solution A1, for 15 min at 0.1ml/min
15.0 Valve at Inject position (loading column), 100% Solution A1, for 100 min at 0.1 ml/min
115.0 Wash with A1 for 6 min at 0.1 ml/min
121.0 Start gradient B1 from 0% to 10% in 2 min at 0.1ml/min
123.0 Gradient B1 from 10% to 25% in 17 min at 0.1ml/min
140.0 Gradient B1 from 25% to 100% in 5 min at 0.1ml/min
145.0 Gradient B1 at 100% for 26 min at 0.1ml/min
171.0 Gradient B1 at 0%
240.0 End of Run

3. Method of LC-Spotting Run

Solution A: 5% acetonitrile. 0.1% TFA, 95% H2O
Solution B: 95% acetonitrile. 0.1% TFA, 5% H2O

0.00 Load trap column with LoadingPump at 40ul/min for 30 min, ValveLeft at 10-1 position, 5%B, 95%A
30.0 ValveLeft at 1-2 position, MicroPump at 2ul/min, for 10 min Gradient of B from 5% to 12%
40.0 %B from 12 to 20 in 3 min, MicroPump 2ul/min
43.0 %B from 20 to 40 in 27 min, MicroPump at 2 ul/min
50.0 Probot turns on
50.1 Probot turns off
70.0 %B from 40 to 40 in 5 min, MicroPump at 2 ul/min
75.0 %B from 40 to 90 in 5 min, MicroPump at 2 ul/min
80.0 %B from 90 to 90 in 10 min, MicroPump at 2 ul/min
90.0 %B from 90 to 5 in 5 min, MicroPump at 2 ul/min
95.0 ValveLeft turns to 10-1 POSOTION
111.0 Probot on
111.1 Probot off
150.0 LoadingPump flow at 0ul/min, MicroPump at 0 ul/min
150.1 End of Run

Appendix 2

Check List

StratumXX
022608
Work Flow Chart
Serum01 Serum02 SerumPL
Depletion Depletion Depletion
Digestion Digestion Digestion
(1/3)iTRAQ114 (1/3)iTRAQ115 (1/3)iTRAQ116
(5/6)Mixture
SCX fractionation into F1 to F6
MALDI/TOF/TOF
Each plate generates 192x25=4800 final spectra
One stratum generates 4800x6=28800
iTRAQ triplet data
C18 RPLC to 192 spots MALDI plate
C18 RPLC to 192 spots MALDI plate
C18 RPLC to 192 spots MALDI plate
C18 RPLC to 192 spots MALDI plate
C18 RPLC to 192 spots MALDI plate
C18 RPLC to 192 spots MALDI plate
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
1. Prepare the following solutions freshly
• Buffer A1: PBS. Dissolve 1 bag of sigma PBS powder in H2O and make up to 1000 ml with H2O
• Buffer B1: Stripping buffer: Add 20 ml of 10X stripping buffer to 180 ml of H2O, mix
• Buffer A2: Neutralization buffer: Add 20 ml of 10X neutralization buffer to 180 ml of H2O, mix
2. Wash the pump heads with 20% ethanol
3. Turn on AKTA system and computer
4. Connect UV cell, conductivity meter to the correct position

5. Wash the pH meter tip with H2O, connect to the pH cell
6. Connect the correct loop (250 ul) to the correct position, wash the injection hole and check the hole size for
compatibility with syringe needle
7. Connect the collection tubing directly to the collection tip, bypassing the accumulator.
8. Make sure that the collection path is not clogged. Wipe the Syncdrop with H2O to clean up any stain on its
walls (if the walls are dirty, the SyncDrop message show up and collection will not start). Make sure that the tip
is in the middle and is tightly screwed in.
9. Equilibrate the tubing and pumps with 10 ml of H2O for each of the 4 pumps at 2 ml/min
10. Wash pumps A and B with H2O, using the built in protocol (PumpWashBasic)
11. Wash the loop with 4 ml of H2O at 4 ml/min
12. Stop flow if it is on
13. Replace Inlet A1 liquid with Buffer A1, B1 liquid with BufferB1, and A2 liquid with Buffer A2, degas for 5 min
14. Start a DepletionEquiSystem method, to bring solutions up to Outlets
15. Wash the loop with 4 ml of Buffer A1 at 1 ml/min, switch from Load to Injection position twice to remove any
trapped air bubble
16. Set flow rate to 0.2 ml/min
17. Immediately after moving the depletion column from 4ºC to room temperature, connect the column to the
correct position following the correct flow direction.
18. Swiftly purge the column with Buffer A1 at 1 ml/min, watch the pressure (normal pressure=0.1)
19. Connect the column outlet to the system, watch the pressure (normal pressure=0.1)
20. Run 10 ml of Buffer A1 through the column, watch the pressure (0), UV(50 at 214), pH(7.4), Conductivity(?)
21. Add 1.5 ml tubes to the 12mm collector rack at A1 through A9
22. Thaw the serum samples on ice
23. Wash the 100 ul syringe with Buffer A1 3 times, 100 ul each, wipe clean the needle with Kimwipe
24. Draw 100 ul of Buffer A1 to the syringe, purge the air bubbles
25. Assure that the valve is at Load position
26. Inject the syringe content to the loop slowly. Make sure there is no leak at the needle tip and observe the liquid
drop in the waste tank. Leave the syringe there
27. Double-check the above items
28. Make a blank depletion run with Buffer A1 that is injected
29. Go to File\Run\Rohan\DepletionRunMethod, click Start, observe the whole run especially at every check point.
30. Most importantly, the collector is on and the collection starts at the right time and in the right tubes. Observe
the spectrum
31. After run, examine that each fraction contains 500 ul of run thru
32. Discard the collections from the blank run. Change to new tubes
33. Spin down the Stratum38-PL-2

34. Add 80 ul of Buffer A1, mix and spin at full speed (13,000 rpm) in a microfuge for 5 min
Depletion/Digestion Run Check List
StratumXX
022608
Depletion Run Check List (page 2), Sample__StratumXX
36. with the 100 ul syringe, take 100 µl. Wipe the needle
37. inject the 100 ul of serum to the loop
38. leave the syringe at inject position
39. Go to File\Run\Rohan\DepletionMethodRun, click Start, observe the whole run especially at every check point.
40. Most importantly, the collector is on and the collection starts at the right time and in the right tubes. Observe the
spectrum
41. After run, examine that each fraction contains 500 ul of run thru
42. collect the fractions from A3 to A8, including A3 and A8 (total is 2.5ml)
43. designate the tube as DP 090101
44. Label three spin ultrafiltration concentrators as conc05-1-2, Conc05-2-2 and Conc05-pool-2, separately.
45. Add 4 ml of 5% Triton-X100 (5k cut off) to each tube. Leave them on bench for more than 30 min
46. Spin down Stratum 39-PL-2
47. Add 80 ul of Buffer A1, mix and spin at full speed for 5 min
48. with the 100 ul syringe, take 100 ul. Wipe the needle
49. inject the 100 ul of serum to the loop
50. leave the syringe the at inject position
51. Go to File\Run\Rohan\DepletionMethodRun, click Start, observe the whole run especially at every check point.
52. Most importantly, the collector is on and the collection starts at the right time and in the right tubes. Observe the
spectrum
53. After run, examine that each fraction contains 500 ul of run thru
54. Collect the fractions from A3 to A8, including A3 and A8 (total is 2.5ml)
55. Designate the tube as Dep 09-2-1
56. Repeat steps 34 to 42 for sample 09PL01
57. Designate the tube as DP09PL01
58. Spin down the three collection tubes
59. Rinse the three spin concentrator from step (45) thoroughly with H2O
60. Transfer the 3 Dep samples to the corresponding concentrators
61. Spin the concentrators (+samples) at 4000g for 40 min first (vol. reduce to 80 ul); mix in 3 ml of 50 mM TEAB
buffer.
62. Spin again at 4,000g for 40 min. Volume must reduce to less than 80ul. Spin once more if needed.
63. Transfer the concentrates individually to three 500-ul tubes labeled iTRAQ090101, 090201, and 09PL01
64. If the volume in the any tube is less than 80, add 50 mM TEAB buffer to make up the volume to 80 ul
65. Add 20 ul of 100% ACN to each tube

66. Dissolve one vial of trypsin in 20 ul of 50mM TEAB with Add 5.5 ul of this reconstituted trypsin solution to each
sample
67. Vortex and spin down
68. Incubate at 37ºC overnight, shaking at 400rpm.
69. Store the digest at 4ºC if not labeling directly.
Day 2 continues on next page
lM1
Slide 4
lM1 This is newly added.
LIYUAN, 2007-8-10
Depletion Run Check List (page 3), Sample: StratumXX
71. From -80C freezer Box A3.3, Move iTRAQ reagent (Lot No. 0710076, Foster City, CA 94404) 114, 115 and 116, and
100% EtOH to room tesmprature, leave on bench for 10 min, spin briefly (10 sec in a microfuge) to collect
the content to the bottom of the tubes.
72. Add swiftly 105 ul of EtOH to each vial, vortex for 30s. Spin down briefly.
73. Dilute 6 ul of the stock dual standard peptide (des-Arg1-Bradykinin Mr=904.5, ACTH18-39, Mr= 2465.2) with
24 ul of 50 mM TEAB, mix well.
74. Prepare three 500ul tubes, label them as pep114, pep115 and pep116. Add 3 ul of iTRAQ reagents to each
corresponding tube
75. Add 1 ul of the diluted standard peptide to each tube, mix and spin
76. Incubate at room temperature for 45 min
77. Spin the digests briefly (10 sec. in a microfuge), add 6 ul of H2O to stop reaction.
78. Take 3 ul from each into a new 500 ul tube, mix, add 9 ul of ready matrix, mix
79. Load to spot on 4700 plate, do MS and MSMS scan to determine the labeling efficiency and the distribution
of the reporter ions.
80. If labeling test passes, contunue the labeling with the serum digest by add 40 ul of digest to each
designated iTRAQ vial. Mix, spin, incubate overnight.
81. Store the EtOH at -80ºC with the Kit in Box A1.1.
82. Stop the reaction by add 420 ul of H2O to each tube (total 560 ul in each tube), spin to pellet the precipitation
83. Take 466 ul of each to a 50 ml tube
84. Add 4,300 ul of SCXA1 solution , mix gently, test pH with paper. pH should be at 3.0
85. Save the rest of 94 ul at -80ºC freezer Box C2.3. at position A5, A6, A7.
86. Subject the diluted mixture to SCX fractionation as the following
lM2
Slide 5
lM2 This is newly added
LIYUAN, 2007-8-10
SCX Run Check List
Sample__StratumXX
Date_____020608

1. Purge the pump heads with 25% ethanol using a 50 ml syringe to remove air bubble
2. Degas the H2O for more than 5 min
3. Switch inlets to H2O
4. Connect UV cell, conductivity meter to the correct position
5. Wash the pH meter tip with H2O, connect to the pH cell
6. Connect the correct loop to the correct position, wash the injection hole and check the hole size for compatibility with syringe needle
7. Connect the collection tubing directly to the collection tip, bypassing the accumulator.
8. Make sure that the collection path is not clogged. Wipe the Syncdrop with H2O to clean up any stain on its walls (if the walls are dirty, the SyncDrop mesagse show up and collection will not start). Make sure that the tip is in the middle and is tightly screwed in.
9. Equilibrate the tubing and pumps with 10 ml of H2O for each of the 4 pumps at 2 ml/min
10. Wash pumps A and B with H2O, using the built in protocol (PumpWashBasic)
11. Wash the loop with 4- folds loop size (8 ml) of H2O at 4 ml/min
12. Stop flow if it is on
13. Replace Inlet A1 liquid with SCXA1 and B1 liquid with SCXB1, degas for 5 min
14. Start a SCXEquiSystem method, to bring solutions up to Outlets
15. Wash the loop with 40 ml of SCXA1 at 4 ml/min, switch from Load to Injection position twice
to remove any trapped air bubble
16. Set flow rate to 0.1ml/min
17. Connect the SCX Precolumn, watch the pump pressure (0.9 normal pressure)
18. Connect the SCX polyA column to the precolumn, watch the pressure (3.2 to 3.5 normal pressure)
19. Connect the column outlet to the system, watch the pressure (3.2 to 3.5 normal pressure)
20. Run 2 ml of SCX A1 through the columns, watch the pressure, UV, pH, Conductivity
21. Add 1.5 ml tubes to the following position on the 12mm collector rack: A1, A2, A3, D1 to D15,
E1, E2, G1 to G15, H1 to H9
22. Bring the iTRAQ labeled digest mixture to room temperature, vortex, spin 1 min at full speed
23. Collect the 450 ul supernatant to 4 ml of SCXA1 in a 50ml tube, mix well gently
24. Wash the 10 ml syringe with SCX A1 3 times, 6 ml each, wipe clean the needle with Kimwipe
25. Draw all 4.50ml sample to the syringe, purge the air bubbles
26. Assure that the valve is at Load position
27. Inject the sample to the loop, slowly but firmly. Make sure there is no leak at the needle tip and observe the liquid drop in the waste tank. Leave the syringe there
28. Double-check the above items
29. Go to File\Run\SCX, click Start, observe the whole run especially at every check point.
30. Most importantly, the collector is on and the collection starts at the right time and in the right
tubes. Observe the conductivity change when elution begins
31. After run, examine that each fraction contains 100 ul of elute
32. Pool fractions D8 to D13 into the D8 tube. Label the vial as S05B_F1
33. Pool fractions D14 to E1 into the E2 tube. Label the vial as S05B_F2
34. Pool fractions E2 to G2 into the G3 tube. Label the vial as S05B_F3
35. Pool fractions G3 to G6 into the G6 tube. Label the vial as S05B_F4

36. Pool fractions G7 to G10 into the G9 tube. Label the vial as S05B_F5
37. Pool fractions G11 to H3 into the G12 tube. Label the vial as S05B_F6
38. Store at 4°C for next day's SpeedVac processing.
Pooling
according to
absorbance at
214
U3000 LC Run Check List
Sample_StratumXX
Date__010208____
Time_____
1. Move SCX fractions from 4°C to room temperature
2. Spin down drops on tube side
3. Prepare the SpeedVac, wait until the vacuum reach the proper level (0.1 atm)
4. Place the tubes balanced in the centrifuge
5. Start Drying process. Stop and check level of sample every 10 min until the sample volume is less than 100 ul
6. Reconstitute the sample volume to 100 with H2O using a 200 ul pipetman
7. Add to each fraction 1 ul of LC standard peptides (peptide 1900, middle, and peptide 1826, late, -80C, BOX C2.1.) which
are reconstituted in 20 ul of 50% ACN and 0.1%TFA
8. Add to each fraction 1 ul of 10% TFA, mix and spin
9. Label 6 U3000 autosampler 250ul vials as SCX05BF1 to SCX05BF6
10. Transfer the fractions to corresponding vial, tape the vial gently to remove trapped air bubbles in vials
11. Place the vials to designated positions in autosampler rack
12. Take 1 vial of ready to use Agilent CHCA matrix (6 mg/ml). Add 3 ul of TFA, mix.
13. Transfer 2 ml to the matrix bottle of the uCarrier dosage unit
14. Add 8 ul of the MS internal standard peptides (bradykinin and ACTH), mix by pipeting
15. Place the matrix bottle in the bottle holder in the dosage unit.
16. Manually purge the syringe, the valves and the spotting needle with the matrix by 5 strokes, be sure no air bubble in the
syringe and be sure to switch the valve position prior to each operation
17. Place six disposable plate inserts in the uCarrier plate holder, write down the plate number in correct order
18. Align the well positions
19. Open the application file, and start application
20. Notice the message at the bottom of the application file as "waiting for the signal from the closure"
21. Prepare fresh solutions A and B for HPLC
22. Purge the pumps
23. Change a new trap column
24. Fill the autosampler wash liquid with solution A, be sure no air bubble in the autosampler syringe
25. Fill the R1 bottle with solution A (used for blank runs)
26. Fill the valve washing liquid bottle with H2O
27. Turn on UV 214 and 254 nm, turn on data acquisition, watch the UV reading (650 mAu at 214 nm)

28. Set flow rat at 40 ul/min for loading pump and 2.0 ul/min for micropump
29. Observe the pressures (90 Value)
30. Start a blank run with R1 solution, 95ul ulPickup injection mode, observe the background
31. Estimate the flow rate at the outlet of UV meter (650 mAu at 214 nm)
32. Connect the outlet of UV to the uCarrier tubing, make sure that liquid comes out of the spotting needle and the pump
pressures are not changed
33. Open the sequence editing page, set up a blank run before each sample run
34. Name the blank runs as blankR1, and the sample runs as LC-SC05BF1 to LC_SC05BF6
35. Make sure the correct vial positions for blank and samples are selected
36. Make sure set injection volume to 95ul
37. Make sure the correct program files are chosen for blank and samples
38. Save the sequence in a new folder under Rohan folder. Name the new folder as LC-SCX05B. Name the sequence file as
LC-SCX05B, too
39. Final check: no liquid leak, LC-Spotting tubing connection, nothing block the uCarrier platform from moving, uCarrier
power on, syringe filled with matrix, plates are in position
40. Start batch, watch any warning message, OK to start
41. Check the run periodically
If necessary (or every 2 weeks),
Clean up the Tof-Tof at this step.
MS/MS analysis ckeck list
1. Before inserting the plate into the autoloader, record the plate bar code and the corresponding fraction and slot numbers.
2. Create a spot set for each plate in LC/MALDI mode
3. Map the spot set to the corresponding plate number and slot number.
4. Load plate to source chamber to align the plate position
5. Test the acquisition and processing method. First test the calibration standards, make sure that both MS1 and MS2 calibration pass the criteria. Then test the sample, spots B10, D10, F10 and H10. Take 3 MS1 peaks (representing high, medium, and low intensity peaks) for MS2 scan. If total intensity is low, increase the laser power.
– MS1: yansen\BCPP\Methods\MS1_Positive_02May2007
– MS1: yansen\BCPP\Methods\MS1_Default_Processing_02May2007
– MS2: yansen\BCPP\Methods\MS2_Positive_02May2007
– MS2: yansen\BCPP\Methods\MS2_Default_Processing_02May2007
6. Choose the methods from yansen\BCPP\Methods for the automatic runs.
7. Submit the Spot Set Job to queue run, observe the validation process.
8. Switch the acquisition mode from interactive to queue run mode.
9. Start the queue run.
10. Observe the beginning of the automatic run, pay attention to the signals.

Stratum__StratumXX
Date__010808__

Data Processing Check List

1. After the TOF/TOF scan, select 116 signals to draw a dot-plot against retention time. If 116 distribution doesn't represent LC spectrum, repeat scanning the abnormal area.
2. Using the ABI data management tool, export the selected ms/ms data files to the drive D:yansen\StratumXX.. as T2D files. Export repeat runs as well.
3. Copy the exported files to the external drive provided by the Epidemiology Dept.
4. Backup the data files to another external hard drive for data storage

Appendix 3

Proteins identified (based on hit number of 500)

Rank Protein Name
1 (P01024) Complement C3 precursor [Contains: C3a anaphylatoxin]
2 (P01023) Alpha-2-macroglobulin precursor (Alpha-2-M)
3 (P01028) Complement C4 precursor [Contains: C4a anaphylatoxin; C4b]
4 (P00450) Ceruloplasmin precursor (EC 1.16.3.1) (Ferroxidase)
5 (P02790) Hemopexin precursor (Beta-1B-glycoprotein)
6 (P04114) Apolipoprotein B-100 precursor (Apo B-100) [Contains: Apolipoprotein B-48 (Apo B-48)]
7 (P02751) Fibronectin precursor (FN) (Cold-insoluble globulin) (CIG)
8 (P19823) Inter-alpha-trypsin inhibitor heavy chain H2 precursor (ITI heavy chain H2) (Inter-alpha-i
9 (P00751) Complement factor B precursor (EC 3.4.21.47) (C3/C5 convertase) (Properdin factor B) (Glyc
10 (P04217) Alpha-1B-glycoprotein precursor (Alpha-1-B glycoprotein)
11 (P06727) Apolipoprotein A-IV precursor (Apo-AIV) (ApoA-IV)
12 (Q14624) Inter-alpha-trypsin inhibitor heavy chain H4 precursor (ITI heavy chain H4) (Inter-alpha-i
13 (P01042) Kininogen precursor (Alpha-2-thiol proteinase inhibitor) [Contains: Bradykinin (Kallidin I
14 (P08603) Complement factor H precursor (H factor 1)
15 (P01011) Alpha-1-antichymotrypsin precursor (ACT)
16 (P02774) Vitamin D-binding protein precursor (DBP) (Group-specific component) (Gc-globulin) (VDB)
17 (P01008) Antithrombin-III precursor (ATIII) (PRO0309)
18 (P19827) Inter-alpha-trypsin inhibitor heavy chain H1 precursor (ITI heavy chain H1) (Inter-alpha-i
19 (P20742) Pregnancy zone protein precursor
20 (P04196) Histidine-rich glycoprotein precursor (Histidine-proline rich glycoprotein) (HPRG)
21 (P00747) Plasminogen precursor (EC 3.4.21.7) [Contains: Angiostatin]
22 (P04004) Vitronectin precursor (Serum spreading factor) (S-protein) (V75) [Contains: Vitronectin V6
23 (P43652) Afamin precursor (Alpha-albumin) (Alpha-Alb)
24 (P01031) Complement C5 precursor [Contains: C5a anaphylatoxin]
25 (P02753) Plasma retinol-binding protein precursor (PRBP) (RBP) (PRO2222)
26 (P02743) Serum amyloid P-component precursor (SAP) (9.5S alpha-1-glycoprotein)
27 (P00736) Complement C1r subcomponent precursor (EC 3.4.21.41) (Complement component 1, r subcompone
28 (P00734) Prothrombin precursor (EC 3.4.21.5) (Coagulation factor II)
29 (P05546) Heparin cofactor II precursor (HC-II) (Protease inhibitor leuserpin 2) (HLS2)
30 (P02775) Platelet basic protein precursor (PBP) (Small inducible cytokine B7) (CXCL7) (Leukocyte-de
31 (P01019) Angiotensinogen precursor [Contains: Angiotensin I (Ang I); Angiotensin II (Ang II); Angio
32 (P02768) Serum albumin precursor
33 (P02749) Beta-2-glycoprotein I precursor (Apolipoprotein H) (Apo-H) (B2GPI) (Beta(2)GPI) (Activated
34 (P03952) Plasma kallikrein precursor (EC 3.4.21.34) (Plasma prekallikrein) (Kininogenin) (Fletcher
35 (P02765) Alpha-2-HS-glycoprotein precursor (Fetuin-A) (Alpha-2-Z-globulin) (Ba-alpha-2-glycoprotein
36 (P02748) Complement component C9 precursor
37 (O75636) Ficolin 3 precursor (Collagen/fibrinogen domain-containing protein 3) (Collagen/fibrinogen
38 (P02760) AMBP protein precursor [Contains: Alpha-1-microglobulin (Protein HC) (Complex-forming glyc
39 (P04003) C4b-binding protein alpha chain precursor (C4bp) (Proline-rich protein) (PRP)
40 (P06396) Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin) (AGEL)
41 (Q06033) Inter-alpha-trypsin inhibitor heavy chain H3 precursor (ITI heavy chain H3) (Inter-alpha-i
42 (P02746) Complement C1q subcomponent, B chain precursor
43 (P00738) Haptoglobin precursor
44 (P01009) Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor) (Alpha-1-antiproteinase) (PRO06
45 (P01871) Ig mu chain C region
46 (P09871) Complement C1s subcomponent precursor (EC 3.4.21.42) (C1 esterase)
47 (Q8TE73) Ciliary dynein heavy chain 5 (Axonemal beta dynein heavy chain 5) (HL1)
48 (P02766) Transthyretin precursor (Prealbumin) (TBPA) (TTR) (ATTR)
49 (P07360) Complement component C8 gamma chain precursor
50 (P07358) Complement component C8 beta chain precursor (Complement component 8 beta subunit)
51 (P29622) Kallistatin precursor (Kallikrein inhibitor) (Protease inhibitor 4)
52 (P02649) Apolipoprotein E precursor (Apo-E)
53 (P39194) Alu subfamily SQ sequence contamination warning entry
54 (O95983) Methyl-CpG-binding domain protein 3 (Methyl-CpG binding protein MBD3)

55 (P35858) Insulin-like growth factor binding protein complex acid labile chain precursor (ALS)
56 (P02656) Apolipoprotein C-III precursor (Apo-CIII) (ApoC-III)
57 (P07996) Thrombospondin-1 precursor
58 (Q9NRC6) Spectrin beta chain, brain 4 (Spectrin, non-erythroid beta chain 4) (Beta-V spectrin) (BSP
59 (P39189) Alu subfamily SB sequence contamination warning entry
60 (P35442) Thrombospondin-2 precursor
61 (P15169) Carboxypeptidase N catalytic chain precursor (EC 3.4.17.3) (Lysine carboxypeptidase) (Argi
62 (Q9Y490) Talin 1
63 (Q08170) Splicing factor, arginine/serine-rich 4 (Pre-mRNA splicing factor SRP75) (SRP001LB)
64 (P10909) Clusterin precursor (Complement-associated protein SP-40,40) (Complement cytolysis inhibit
65 (P06753) Tropomyosin alpha 3 chain (Tropomyosin 3) (Tropomyosin gamma) (hTM5)
66 (Q99661) Kinesin-like protein KIF2C (Mitotic centromere-associated kinesin) (MCAK) (Kinesin-like pr
67 (Q9Y6E0) Serine/threonine-protein kinase 24 (EC 2.7.1.37) (STE20-like kinase MST3) (MST-3) (Mammali
68 (P39191) Alu subfamily SB2 sequence contamination warning entry
69 (O43781) Dual-specificity tyrosine-phosphorylation regulated kinase 3 (EC 2.7.1.-)
70 (O95479) GDH/6PGL endoplasmic bifunctional protein precursor [Includes: Glucose 1-dehydrogenase (EC
71 (P53396) ATP-citrate synthase (EC 2.3.3.8) (ATP-citrate (pro-S-)-lyase) (Citrate cleavage enzyme)
72 (P12111) Collagen alpha 3(VI) chain precursor
73 (O60237) Protein phosphatase 1 regulatory subunit 12B (Myosin phosphatase targeting subunit 2) (Myo
74 (Q96F44) Tripartite motif protein 11 (EC 6.3.2.-) (RING finger protein 92) (BIA1 protein)
75 (Q96T23) Hepatitis B virus x associated protein (HBV pX associated protein 8) (Remodeling and spaci
76 (O94973) Adapter-related protein complex 2 alpha 2 subunit (Alpha-adaptin C) (Adaptor protein compl
77 (P02647) Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)
78 (O60741) Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 1 (Brain cycl
79 (P07205) Phosphoglycerate kinase, testis specific (EC 2.7.2.3)
80 (Q8IYF1) RNA polymerase II transcription factor SIII subunit A2 (Elongin A2) (EloA2) (Transcription
81 (O75874) Isocitrate dehydrogenase [NADP] cytoplasmic (EC 1.1.1.42) (Oxalosuccinate decarboxylase) (
82 (P06400) Retinoblastoma-associated protein (PP110) (P105-RB) (RB)
83 (Q9Y224) Protein C14orf166 (CGI-99)
84 (Q8IZT6) Abnormal spindle-like microcephaly-associated protein (Abnormal spindle protein homolog) (
85 (Q8WZ75) Roundabout homolog 4 precursor (Magic roundabout) (UNQ421/PRO3674)
86 (Q9NVW2) RING finger protein 12 (LIM domain interacting RING finger protein) (RING finger LIM domai
87 (Q15848) Adiponectin precursor (Adipocyte, C1q and collagen domain containing protein) (30 kDa adip
88 (O95425) Supervillin (Archvillin) (p205/p250)
89 (O14757) Serine/threonine-protein kinase Chk1 (EC 2.7.1.37)
90 (Q14152) Eukaryotic translation initiation factor 3 subunit 10 (eIF-3 theta) (eIF3 p167) (eIF3 p180
91 (Q9HCS7) XPA-binding protein 2 (HCNP protein) (PP3898)
92 (O75694) Nuclear pore complex protein Nup155 (Nucleoporin Nup155) (155 kDa nucleoporin)
93 (Q8TEU7) Rap guanine nucleotide exchange factor 6 (PDZ domain containing guanine nucleotide exchang
94 (Q96JA1) Leucine-rich repeats and immunoglobulin-like domains protein 1 precursor (LIG-1)
95 (Q9UHF7) Zinc finger transcription factor Trps1 (Zinc finger protein GC79) (Tricho-rhino-phalangeal
96 (P11532) Dystrophin
97 (P00451) Coagulation factor VIII precursor (Procoagulant component) (Antihemophilic factor) (AHF)
98 (Q03468) DNA excision repair protein ERCC-6 (Cockayne syndrome protein CSB)
99 (Q9NYQ6) Cadherin EGF LAG seven-pass G-type receptor 1 precursor (Flamingo homolog 2) (hFmi2)
100 (Q9UJV3) Midline 2 protein (Midline defect 2) (Tripartite motif protein 1) (Midin 2) (RING finger p
101 (O95782) Adapter-related protein complex 2 alpha 1 subunit (Alpha-adaptin A) (Adaptor protein compl
102 (P11021) 78 kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding pr
103 (Q70EK9) Ubiquitin carboxyl-terminal hydrolase 51 (EC 3.1.2.15) (Ubiquitin thiolesterase 51) (Ubiqu
104 (Q96KN2) Glutamate carboxypeptidase-like protein 2 precursor (CNDP dipeptidase 1)
105 (Q93073) Protein KIAA0256
106 (Q9NQ55) Suppressor of SWI4 1 homolog (Ssf-1) (Peter Pan homolog)
107 (Q99728) BRCA1-associated RING domain protein 1 (BARD-1)
108 (Q14571) Inositol 1,4,5-trisphosphate receptor type 2 (Type 2 inositol 1,4,5-trisphosphate receptor
109 (Q9WJR5) HERV-K_19q12 provirus ancestral Pol protein (HERV-K(C19) Pol protein) [Includes: Reverse t
110 (P14625) Endoplasmin precursor (94 kDa glucose-regulated protein) (GRP94) (gp96 homolog) (Tumor rej
111 (O75533) Splicing factor 3B subunit 1 (Spliceosome associated protein 155) (SAP 155) (SF3b155) (Pre
112 (Q13029) PR-domain zinc finger protein 2 (Retinoblastoma protein-interacting zinc-finger protein) (
113 (O75417) DNA polymerase theta (EC 2.7.7.7) (DNA polymerase eta)
114 (Q9P2R3) Ankyrin repeat and FYVE domain protein 1 (Ankyrin repeats hooked to a zinc finger motif)
115 (P23229) Integrin alpha-6 precursor (VLA-6) (CD49f)
116 (Q13315) Serine-protein kinase ATM (EC 2.7.1.37) (Ataxia telangiectasia mutated) (A-T, mutated)
117 (Q9BUG6) Zinc finger and SCAN domain containing protein 5 (Zinc finger protein 495)
118 (Q03001) Bullous pemphigoid antigen 1 isoforms 1/2/3/4/5/8 (230 kDa bullous pemphigoid antigen) (BP

119 (O95970) Leucine-rich glioma-inactivated protein 1 precursor (Epitempin 1) (UNQ775/PRO1569)
120 (O95347) Structural maintenance of chromosome 2-like 1 protein (Chromosome-associated protein E) (h
121 (P05156) Complement factor I precursor (EC 3.4.21.45) (C3B/C4B inactivator)
122 (Q99973) Telomerase protein component 1 (Telomerase-associated protein 1) (Telomerase protein 1) (p
123 (Q9NSA2) Potassium voltage-gated channel subfamily D member 1 (Voltage-gated potassium channel subu
124 (P13535) Myosin heavy chain, skeletal muscle, perinatal (MyHC-perinatal)
125 (Q9NZV8) Potassium voltage-gated channel subfamily D member 2 (Voltage-gated potassium channel subu
126 (Q14289) Protein tyrosine kinase 2 beta (EC 2.7.1.112) (Focal adhesion kinase 2) (FADK 2) (Proline-
127 (Q9UQ05) Potassium voltage-gated channel subfamily H member 4 (Voltage-gated potassium channel subu
128 (Q9UGM5) Fetuin-B precursor (IRL685) (16G2)
129 (P42681) Tyrosine-protein kinase TXK (EC 2.7.1.112)
130 (O60469) Down syndrome cell adhesion molecule precursor (CHD2)
131 (P49895) Type I iodothyronine deiodinase (EC 1.97.1.10) (Type-I 5'deiodinase) (DIOI) (Type 1 DI) (5
132 (P02042) Hemoglobin delta chain
133 (Q8WWQ8) Stabilin 2 precursor (FEEL-2 protein) (Fasciclin EGF-like laminin-type EGF-like and link d
134 (Q96L93) Kinesin-like motor protein C20orf23 (Sorting nexin 23)
135 (Q9GZU5) Nyctalopin precursor
136 (P20848) Alpha-1-antitrypsin-related protein precursor
137 (Q9BXT8) RING finger protein 17
138 (O76039) Serine/threonine-protein kinase 9 (EC 2.7.1.37) (Cyclin-dependent kinase-like 5)
139 (P56715) Oxygen-regulated protein 1 (Retinitis pigmentosa RP1 protein) (Retinitis pigmentosa 1 prot
140 (P27169) Serum paraoxonase/arylesterase 1 (EC 3.1.1.2) (EC 3.1.8.1) (PON 1) (Serum aryldialkylphosp
141 (P20711) Aromatic-L-amino-acid decarboxylase (EC 4.1.1.28) (AADC) (DOPA decarboxylase) (DDC)
142 (Q86VI3) Ras GTPase-activating-like protein IQGAP3
143 (O15056) Synaptojanin 2 (EC 3.1.3.36) (Synaptic inositol-1,4,5-trisphosphate 5-phosphatase 2)
144 (Q13094) Lymphocyte cytosolic protein 2 (SH2 domain-containing leucocyte protein of 76 kDa) (SLP-76 tyrosine
145 (O60563) Cyclin T1 (Cyclin T) (CycT1)
146 (Q9UIF8) Bromodomain adjacent to zinc finger domain 2B (hWALp4)
147 (Q14980) Nuclear mitotic apparatus protein 1 (NuMA protein) (SP-H antigen)
148 (P25054) Adenomatous polyposis coli protein (APC protein)
149 (P39190) Alu subfamily SB1 sequence contamination warning entry
150 (Q96RT6) Protein cTAGE-2
151 (Q15431) Synaptonemal complex protein 1 (SCP-1 protein)
152 (Q14683) Structural maintenance of chromosome 1-like 1 protein (SMC1alpha protein) (SB1.8/DXS423E p
153 (Q12923) Tyrosine-protein phosphatase, non-receptor type 13 (EC 3.1.3.48) (Protein-tyrosine phospha
154 (P20138) Myeloid cell surface antigen CD33 precursor (gp67) (Siglec-3)
155 (Q8WXH0) Nesprin 2 (Nuclear envelope spectrin repeat protein 2) (Syne-2) (Synaptic nuclear envelope
156 (Q9Y6N7) Roundabout homolog 1 precursor (H-Robo-1) (Deleted in U twenty twenty)
157 (P01861) Ig gamma-4 chain C region
158 (P02747) Complement C1q subcomponent, C chain precursor
159 (Q9Y4G6) Talin 2
160 (P43627) Killer cell immunoglobulin-like receptor 2DL2 precursor (MHC class I NK cell receptor) (Na
161 (P42566) Epidermal growth factor receptor substrate 15 (Protein Eps15) (AF-1p protein)
162 (Q9ULB1) Neurexin 1-alpha precursor (Neurexin I-alpha)
163 (Q9ULJ7) Hypothetical protein KIAA1223 (Fragment)
164 (Q9NQS7) Inner centromere protein
165 (Q15078) Cyclin-dependent kinase 5 activator 1 precursor (CDK5 activator 1) (Cyclin-dependent kinas
166 (Q01082) Spectrin beta chain, brain 1 (Spectrin, non-erythroid beta chain 1) (Beta-II spectrin) (Fo
167 (P10721) Mast/stem cell growth factor receptor precursor (EC 2.7.1.112) (SCFR) (Proto-oncogene tyro
168 (Q9Y4K1) Absent in melanoma 1 protein
169 (Q9UPN3) Microtubule-actin crosslinking factor 1, isoforms 1/2/3/5 (Actin cross-linking family prot
170 (Q8WXK8) Bullous pemphigoid antigen 1, isoform 7 (Bullous pemphigoid antigen) (BPA) (Hemidesmosomal
171 (Q06413) Myocyte-specific enhancer factor 2C
172 (P36955) Pigment epithelium-derived factor precursor (PEDF) (EPC-1)
173 (P36980) Complement factor H-related protein 2 precursor (FHR-2) (H factor-like protein 2) (H facto
174 (Q14896) Myosin-binding protein C, cardiac-type (Cardiac MyBP-C) (C-protein, cardiac muscle isoform
175 (Q9P2F8) Signal-induced proliferation-associated 1 like protein 2 (Fragment)
176 (Q9HCS5) Band 4.1-like protein 4A (NBL4 protein)
177 (Q9UGU5) High-mobility group protein 2-like 1 (HMGBCG protein)
178 (Q14573) Inositol 1,4,5-trisphosphate receptor type 3 (Type 3 inositol 1,4,5-trisphosphate receptor
179 (P13647) Keratin, type II cytoskeletal 5 (Cytokeratin 5) (K5) (CK 5) (58 kDa cytokeratin)
180 (Q8WXE1) ATR-interacting protein (ATM and Rad3 related interacting protein)
181 (Q8IZU2) WD-repeat protein 17
182 (O14910) LIN-7 homolog A (LIN-7A) (hLin-7) (Mammalian LIN-seven protein 1) (MALS-1) (Vertebrate LIN

183 (Q96I25) Splicing factor 45 (45kDa splicing factor) (RNA binding motif protein 17)
184 (O60229) Huntingtin-associated protein-interacting protein (Duo protein)
185 (Q9BVV6) Protein KIAA0586
186 (Q99828) Calcium and integrin-binding protein 1 (Calmyrin) (DNA-PKcs interacting protein) (Kinase i
187 (O75509) Tumor necrosis factor receptor superfamily member 21 precursor (TNFR-related death recepto
188 (O75376) Nuclear receptor corepressor 1 (N-CoR1) (N-CoR)
189 (P82279) Crumbs protein homolog 1 precursor
190 (P98164) Low-density lipoprotein receptor-related protein 2 precursor (Megalin) (Glycoprotein 330)
191 (Q8NHQ1) Centrosomal protein of 70 kDa (Cep70 protein) (p10-binding protein)
192 (Q9P2G1) Ankyrin repeat and IBR domain containing protein 1 (Fragment)
193 (Q9Y2I7) FYVE finger-containing phosphoinositide kinase (EC 2.7.1.68) (1-phosphatidylinositol-4-pho
194 (P12838) Neutrophil defensin 4 precursor (HNP-4) (HP-4) (Defensin, alpha 4)
195 (O75132) Zinc finger BED domain containing protein 4
196 (O14522) Receptor-type tyrosine-protein phosphatase T precursor (EC 3.1.3.48) (R-PTP-T) (RPTP-rho)
197 (Q9H0D6) 5'-3' exoribonuclease 2 (EC 3.1.11.-) (DHM1-like protein) (DHP protein)
198 (Q9Y2L1) Exosome complex exonuclease RRP44 (EC 3.1.13.-) (Ribosomal RNA processing protein 44) (DIS
199 (P55201) Peregrin (Bromodomain and PHD finger-containing protein 1) (BR140 protein)
200 (Q9UBZ9) DNA repair protein REV1 (EC 2.7.7.-) (Rev1-like terminal deoxycytidyl transferase) (Alpha
201 (Q8IZP9) G-protein coupled receptor 64 precursor (Epididymis-specific protein 6) (He6 receptor)
202 (Q9Y6D5) Brefeldin A-inhibited guanine nucleotide-exchange protein 2 (Brefeldin A-inhibited GEP 2)
203 (O75882) Attractin precursor (Mahogany homolog) (DPPT-L)
204 (O94782) Ubiquitin carboxyl-terminal hydrolase 1 (EC 3.1.2.15) (Ubiquitin thiolesterase 1) (Ubiquit
205 (O75095) Multiple EGF-like-domain protein 3 (Multiple epidermal growth factor-like domains 6) (Frag
206 (Q16787) Laminin alpha-3 chain precursor (Epiligrin 170 kDa subunit) (E170) (Nicein alpha subunit)
207 (Q9H4A3) Serine/threonine-protein kinase WNK1 (EC 2.7.1.37) (Protein kinase with no lysine 1) (Prot
208 (O95104) Splicing factor, arginine/serine-rich 15 (CTD-binding SR-like protein RA4)
209 (O95602) DNA-directed RNA polymerase I largest subunit (EC 2.7.7.6) (RNA polymerase I 194 kDa subun
210 (P07602) Proactivator polypeptide precursor [Contains: Saposin A (Protein A); Saposin B (Sphingolip
211 (Q9H3H1) tRNA isopentenyltransferase, mitochondrial precursor (EC 2.5.1.8) (Isopentenyl-diphosphate
212 (Q9C0H6) Kelch-like protein 4
213 (O14764) Gamma-aminobutyric-acid receptor delta subunit precursor (GABA(A) receptor)
214 (Q92692) Poliovirus receptor related protein 2 precursor (Herpes virus entry mediator B) (HveB) (Ne
215 (O94808) Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 2 (EC 2.6.1.16) (Hexoseph
216 (Q13415) Origin recognition complex subunit 1 (Replication control protein 1)
217 (Q13105) Zinc finger and BTB domain containing protein 17 (Zinc finger protein 151) (Myc-interactin
218 (P67809) Nuclease sensitive element binding protein 1 (Y-box binding protein-1) (Y-box transcriptio
219 (Q14493) Histone RNA hairpin-binding protein (Histone stem-loop binding protein)
220 (P14923) Junction plakoglobin (Desmoplakin III)
221 (P50851) Lipopolysaccharide-responsive and beige-like anchor protein (CDC4-like protein) (Beige-lik
222 (Q96P65) Orexigenic neuropeptide QRFP receptor (G-protein coupled receptor 103) (SP9155) (AQ27)
223 (Q96PK2) Microtubule-actin crosslinking factor 1, isoform 4
224 (Q9UDV7) Zinc finger protein 282 (HTLV-I U5RE binding protein 1) (HUB-1)
225 (Q9Y5Y9) Sodium channel protein type X alpha subunit (Voltage-gated sodium channel alpha subunit Na
226 (Q9C040) Tripartite motif protein 2 (RING finger protein 86)
227 (P08697) Alpha-2-antiplasmin precursor (Alpha-2-plasmin inhibitor) (Alpha-2-PI) (Alpha-2-AP)
228 (P02787) Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal binding globulin) (PR
229 (P02786) Transferrin receptor protein 1 (TfR1) (TR) (TfR) (Trfr) (CD71 antigen) (T9) (p90)
230 (Q96RD0) Olfactory receptor 8B2
231 (O00625) Pirin
232 (O75161) Nephrocystin 4 (Nephroretinin)
233 (Q9UK17) Potassium voltage-gated channel subfamily D member 3 (Voltage-gated potassium channel subu
234 (Q02224) Centromeric protein E (CENP-E protein)
235 (Q9NQT4) Exosome complex exonuclease RRP46 (EC 3.1.13.-) (Ribosomal RNA processing protein 46) (Exo
236 (Q00059) Transcription factor A, mitochondrial precursor (mtTFA) (Mitochondrial transcription facto
237 (Q9Y2T1) Axin-2 (Axis inhibition protein 2) (Conductin) (Axin-like protein) (Axil)
238 (Q96DT6) Cysteine protease APG4C (EC 3.4.22.-) (Autophagy 4 homolog C) (Autophagin-3) (Autophagy-re
239 (Q9Y625) Glypican-6 precursor (UNQ369/PRO705)
240 (O60333) Kinesin-like protein KIF1B (Klp)
241 (Q14790) Caspase-8 precursor (EC 3.4.22.-) (CASP-8) (ICE-like apoptotic protease 5) (MORT1-associat
242 (Q9NZM3) Intersectin 2 (SH3 domain-containing protein 1B) (SH3P18) (SH3P18-like WASP associated pro
243 (Q92630) Dual-specificity tyrosine-phosphorylation regulated kinase 2 (EC 2.7.1.112) (EC 2.7.1.37)
244 (Q9P2L0) WD-repeat protein 35
245 (O15397) Importin 8 (Imp8) (Ran-binding protein 8) (RanBP8)
246 (Q14031) Collagen alpha 6(IV) chain precursor

247 (Q8NF91) Nesprin 1 (Nuclear envelope spectrin repeat protein 1) (Synaptic nuclear envelope protein
248 (O95171) Sciellin
249 (Q9NZJ4) Sacsin
250 (P39188) Alu subfamily J sequence contamination warning entry
251 (Q9Y4X5) Ariadne-1 protein homolog (ARI-1) (Ubiquitin-conjugating enzyme E2-binding protein 1) (Ubc
252 (Q16288) NT-3 growth factor receptor precursor (EC 2.7.1.112) (Neurotrophic tyrosine kinase recepto
253 (O75116) Rho-associated protein kinase 2 (EC 2.7.1.37) (Rho-associated, coiled-coil containing prot
254 (Q9C0C2) 182 kDa tankyrase 1-binding protein
255 (O60503) Adenylate cyclase, type IX (EC 4.6.1.1) (ATP pyrophosphate-lyase 9) (Adenylyl cyclase 9)
256 (Q8TF05) Serine/threonine phosphatase 4 regulatory subunit 1
257 (Q9BV73) Centrosomal protein 2 (Centrosomal Nek2-associated protein 1) (C-NAP1) (Centrosome protein
258 (O95402) Cofactor required for Sp1 transcriptional activation subunit 7 (Transcriptional coactivato
259 (Q9Y264) Angiopoietin-4 precursor (ANG-4) (ANG-3)
260 (P22352) Plasma glutathione peroxidase precursor (EC 1.11.1.9) (GSHPx-P) (Extracellular glutathione
261 (Q00839) Heterogenous nuclear ribonucleoprotein U (hnRNP U) (Scaffold attachment factor A) (SAF-A)
262 (P59910) Testis spermatocyte apoptosis-related gene 6 protein (Testis and spermatogenesis cell rela
263 (Q9HCK0) Zinc finger and BTB domain containing protein 26 (Zinc finger protein 481) (Zinc finger pr
264 (P61129) Zinc finger CCCH type domain containing protein 6
265 (Q13227) G protein pathway suppressor 2 (GPS2 protein)
266 (P63135) HERV-K_1q22 provirus ancestral Pol protein (HERV-K102 Pol protein) (HERV-K(III) Pol protei
267 (Q96ST3) Paired amphipathic helix protein Sin3a
268 (Q9UQM7) Calcium/calmodulin-dependent protein kinase type II alpha chain (EC 2.7.1.123) (CaM-kinase
269 (O15091) Hypothetical protein KIAA0391
270 (Q13620) Cullin homolog 4B (CUL-4B)
271 (Q8WYA0) Carnitine deficiency-associated protein expressed in ventricle 1 (CDV-1 protein)
272 (P28827) Receptor-type tyrosine-protein phosphatase mu precursor (EC 3.1.3.48) (Protein-tyrosine ph
273 (P16615) Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (EC 3.6.3.8) (Calcium pump 2) (SERCA2)
274 (P30414) NK-tumor recognition protein (Natural-killer cells cyclophilin-related protein) (NK-TR pro
275 (P26196) Probable ATP-dependent RNA helicase p54 (Oncogene RCK) (DEAD-box protein 6)
276 (Q9Y3L3) SH3-domain binding protein 1 (3BP-1)
277 (Q9Y4C0) Neurexin 3-alpha precursor (Neurexin III-alpha)
278 (Q9Y6D9) Mitotic spindle assembly checkpoint protein MAD1 (Mitotic arrest deficient-like protein 1)
279 (P50995) Annexin A11 (Annexin XI) (Calcyclin-associated annexin 50) (CAP-50) (56 kDa autoantigen)
280 (O43889) Cyclic-AMP responsive element binding protein 3 (Luman protein) (Transcription factor LZIP
281 (P06307) Cholecystokinins precursor (CCK) [Contains: Cholecystokinin 58 (CCK58); Cholecystokinin 39
282 (P48047) ATP synthase oligomycin sensitivity conferral protein, mitochondrial precursor (EC 3.6.3.1
283 (Q13506) NGFI-A binding protein 1 (EGR-1 binding protein 1) (Transcriptional regulatory protein p54
284 (Q9BZF2) Oxysterol binding protein-related protein 7 (OSBP-related protein 7) (ORP-7)
285 (Q14993) Collagen alpha 1(XIX) chain precursor (Collagen alpha 1(Y) chain)
286 (P05155) Plasma protease C1 inhibitor precursor (C1 Inh) (C1Inh)
287 (P35914) Hydroxymethylglutaryl-CoA lyase, mitochondrial precursor (EC 4.1.3.4) (HMG-CoA lyase) (HL)
288 (P54108) Cysteine-rich secretory protein-3 precursor (CRISP-3) (SGP28 protein)
289 (P17936) Insulin-like growth factor binding protein 3 precursor (IGFBP-3) (IBP-3) (IGF-binding prot
290 (P58340) Myeloid leukemia factor 1 (Myelodysplasia-myeloid leukemia factor 1)
291 (P50991) T-complex protein 1, delta subunit (TCP-1-delta) (CCT-delta) (Stimulator of TAR RNA bindin
292 (O00471) Exocyst complex component Sec10 (hSec10)
293 (Q14721) Potassium voltage-gated channel subfamily B member 1 (Voltage-gated potassium channel subu
294 (Q96F15) GTPase, IMAP family member 5 (Immunity-associated nucleotide 4-like 1 protein) (Immunity-a
295 (Q9UBX3) Mitochondrial dicarboxylate carrier
296 (P06241) Proto-oncogene tyrosine-protein kinase FYN (EC 2.7.1.112) (P59-FYN) (SYN) (SLK)
297 (Q8IWJ2) GRIP and coiled-coil domain-containing protein 2 (Golgi coiled coil protein GCC185) (CTCL
298 (O75791) GRB2-related adaptor protein 2 (GADS protein) (Growth factor receptor binding protein) (GR
299 (O15360) Fanconi anemia group A protein (FACA protein)
300 (P09668) Cathepsin H precursor (EC 3.4.22.16)
301 (O75113) Nedd4-binding protein 1 (N4BP1) (Fragment)
302 (P51826) LAF-4 protein (Lymphoid nuclear protein related to AF4)
303 (Q9UQE7) Structural maintenance of chromosome 3 (Chondroitin sulfate proteoglycan 6) (Chromosome-as
304 (Q04721) Neurogenic locus notch homolog protein 2 precursor (Notch 2) (hN2)
305 (P18206) Vinculin (Metavinculin)
306 (P24821) Tenascin precursor (TN) (Hexabrachion) (Cytotactin) (Neuronectin) (GMEM) (JI) (Miotendinou
307 (Q8TC27) ADAM 32 precursor (A disintegrin and metalloprotease domain 32) (UNQ5982/PRO21340)
308 (P05543) Thyroxine-binding globulin precursor (T4-binding globulin)
309 (Q12789) General transcription factor 3C polypeptide 1 (Transcription factor IIIC-alpha subunit) (T
310 (Q08211) ATP-dependent RNA helicase A (Nuclear DNA helicase II) (NDH II) (DEAH-box protein 9)

311 (P57058) Hormonally up-regulated neu tumor-associated kinase (EC 2.7.1.37) (Serine/threonine-protei
312 (O14862) Interferon-inducible protein AIM2 (Absent in melanoma 2)
313 (Q9UMN6) Myeloid/lymphoid or mixed-lineage leukemia protein 4 (Trithorax homolog 2)
314 (Q02880) DNA topoisomerase II, beta isozyme (EC 5.99.1.3)
315 (P56192) Methionyl-tRNA synthetase (EC 6.1.1.10) (Methionine--tRNA ligase) (MetRS)
316 (P38935) DNA-binding protein SMUBP-2 (Immunoglobulin mu binding protein 2) (SMUBP-2) (Glial factor-
317 (P05164) Myeloperoxidase precursor (EC 1.11.1.7) (MPO)
318 (P31639) Sodium/glucose cotransporter 2 (Na(+)/glucose cotransporter 2) (Low affinity sodium-glucos
319 (Q86YR6) Ankyrin repeat domain protein 21 (POTE protein) (Prostate, ovary, testis expressed protein
320 (O15020) Spectrin beta chain, brain 2 (Spectrin, non-erythroid beta chain 2) (Beta-III spectrin)
321 (O95071) Ubiquitin--protein ligase EDD (EC 6.3.2.-) (Hyperplastic discs protein homolog) (hHYD) (Pr
322 (Q8IVF6) Ankyrin repeat domain protein 18A
323 (P12643) Bone morphogenetic protein 2 precursor (BMP-2) (BMP-2A)
324 (Q8TCX5) Rhophilin 1 (GTP-Rho binding protein 1)
325 (O00198) Activator of apoptosis harakiri (Neuronal death protein DP5) (BH3 interacting domain prote
326 (Q9UHA3) Probable ribosome biogenesis protein RLP24 (Ribosomal protein L24-like) (My024 protein)
327 (P33121) Long-chain-fatty-acid--CoA ligase 1 (EC 6.2.1.3) (Long-chain acyl-CoA synthetase 1) (LACS
328 (P49736) DNA replication licensing factor MCM2 (Minichromosome maintenance protein 2 homolog) (Nucl
329 (O60312) Potential phospholipid-transporting ATPase VA (EC 3.6.3.1) (ATPVA) (Aminophospholipid tran
330 (P17023) Zinc finger protein 19 (Zinc finger protein KOX12)
331 (O00555) Voltage-dependent P/Q-type calcium channel alpha-1A subunit (Voltage-gated calcium channel
332 (O60343) TBC1 domain family member 4
333 (Q9NUP9) LIN-7 homolog C (LIN-7C) (Mammalian LIN-seven protein 3) (MALS-3) (Vertebrate LIN 7 homolo
334 (Q9NVU0) DNA-directed RNA polymerases III 80 kDa polypeptide (EC 2.7.7.6) (RNA polymerase III subun
335 (O14795) Unc-13 homolog B (Munc13-2) (munc13)
336 (P48552) Nuclear factor RIP140 (Nuclear receptor interacting protein 1)
337 (Q9Y463) Dual-specificity tyrosine-phosphorylation regulated kinase 1B (EC 2.7.1.37) (EC 2.7.1.112)
338 (O43474) Kruppel-like factor 4 (Epithelial zinc-finger protein EZF) (Gut-enriched Krueppel-like fac
339 (Q9NQE7) Thymus-specific serine protease precursor (EC 3.4.-.-)
340 (P07814) Bifunctional aminoacyl-tRNA synthetase [Includes: Glutamyl-tRNA synthetase (EC 6.1.1.17) (
341 (Q6W2J9) BCoR protein (BCL-6 corepressor)
342 (P01213) Beta-neoendorphin-dynorphin precursor (Proenkephalin B) (Preprodynorphin) [Contains: Beta-
343 (O00622) CYR61 protein precursor (Cysteine-rich, angiogenic inducer, 61) (Insulin-like growth facto
344 (P26367) Paired box protein Pax-6 (Oculorhombin) (Aniridia, type II protein)
345 (Q9NQ66) 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 1 (EC 3.1.4.11) (Phosphoino
346 (Q9P2K8) Eukaryotic translation initiation factor 2-alpha kinase 4 (EC 2.7.1.37) (GCN2-like protein
347 (P28289) Tropomodulin-1 (Erythrocyte tropomodulin) (E-Tmod)
348 (Q9P2R7) Succinyl-CoA ligase [ADP-forming] beta-chain, mitochondrial precursor (EC 6.2.1.5) (Succin
349 (Q15413) Ryanodine receptor 3 (Brain-type ryanodine receptor) (RyR3) (RYR-3) (Brain ryanodine recep
350 (O14983) Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (EC 3.6.3.8) (Calcium pump 1) (SERCA1)
351 (Q00653) Nuclear factor NF-kappa-B p100/p49 subunits (DNA-binding factor KBF2) (H2TF1) (Lymphocyte
352 (Q6UX41) Butyrophilin-like protein 8 precursor (UNQ702/PRO1347)
353 (O95819) Mitogen-activated protein kinase kinase kinase kinase 4 (EC 2.7.1.37) (MAPK/ERK kinase kin
354 (Q8TF76) Serine/threonine-protein kinase Haspin (EC 2.7.1.37) (Haploid germ cell-specific nuclear p
355 (Q9BZ29) Dedicator of cytokinesis protein 9 (Cdc42 guanine nucleotide exchange factor zizimin 1)
356 (O15270) Serine palmitoyltransferase 2 (EC 2.3.1.50) (Long chain base biosynthesis protein 2) (LCB
357 (Q92698) DNA repair and recombination protein RAD54-like (EC 3.6.1.-) (RAD54 homolog) (hRAD54) (hHR
358 (Q9Y2D4) Exocyst complex component Sec15B
359 (Q9UJF2) Ras GTPase-activating protein nGAP (RAS protein activator like 1)
360 (Q07283) Trichohyalin
361 (Q16513) Protein kinase N2 (EC 2.7.1.37) (Protein kinase C-like 2) (Protein-kinase C-related kinase
362 (O15294) UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit (EC 2.4.1
363 (O43927) Small inducible cytokine B13 precursor (CXCL13) (B lymphocyte chemoattractant) (CXC chemok
364 (Q15057) Centaurin beta 2 (Cnt-b2)
365 (P04275) Von Willebrand factor precursor (vWF) [Contains: Von Willebrand antigen II]
366 (P62244) 40S ribosomal protein S15a
367 (O15393) Transmembrane protease, serine 2 precursor (EC 3.4.21.-)
368 (Q9UPM8) Adapter-related protein complex 4 epsilon 1 subunit (Epsilon subunit of AP-4) (AP-4 adapte
369 (Q8NBK3) Sulfatase modifying factor 1 precursor (C-alpha-formylglycine-generating enzyme 1)
370 (P16157) Ankyrin 1 (Erythrocyte ankyrin) (Ankyrin R)
371 (Q9NR96) Toll-like receptor 9 precursor
372 (P15144) Aminopeptidase N (EC 3.4.11.2) (hAPN) (Alanyl aminopeptidase) (Microsomal aminopeptidase)
373 (Q8IY33) MICAL-like protein 2
374 (P35241) Radixin

375 (Q12816) Trophinin (MAGE-D3 antigen)
376 (P55084) Trifunctional enzyme beta subunit, mitochondrial precursor (TP-beta) (MSTP029) [Includes:
377 (Q9UL51) Potassium/sodium hyperpolarization-activated cyclic nucleotide-gated channel 2 (Brain cycl
378 (Q8NF50) Dedicator of cytokinesis protein 8 (Fragment)
379 (P14616) Insulin receptor-related protein precursor (EC 2.7.1.112) (IRR) (IR-related receptor)
380 (P20929) Nebulin
381 (P01112) Transforming protein p21/H-Ras-1 (c-H-ras)
382 (O15123) Angiopoietin-2 precursor (ANG-2)
383 (O14727) Apoptotic protease activating factor 1 (Apaf-1)
384 (P28715) DNA-repair protein complementing XP-G cells (Xeroderma pigmentosum group G complementing p
385 (Q9BXT6) Potential helicase Mov10l1 (EC 3.6.1.-) (Moloney leukemia virus 10-like protein 1) (MOV10-
386 (Q9UGQ3) Solute carrier family 2, facilitated glucose transporter, member 6 (Glucose transporter ty
387 (Q9H0N0) Ras-related protein Rab-6C (Rab6-like protein WTH3)
388 (P13639) Elongation factor 2 (EF-2)
389 (Q9Y5X9) Endothelial lipase precursor (EC 3.1.1.3) (Endothelial cell-derived lipase) (EDL) (EL)
390 (P27469) Putative lymphocyte G0/G1 switch protein 2
391 (Q9NSI8) SAM-domain protein SAMSN-1 (SAM domain, SH3 domain and nuclear localisation signals protei
392 (Q96A11) Galactose-3-O-sulfotransferase 3 (EC 2.8.2.-) (Gal3ST-3) (Galbeta1-3GalNAc 3'-sulfotransfe
393 (P47985) Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial precursor (EC 1.10.2.2
394 (Q15746) Myosin light chain kinase, smooth muscle and non-muscle isozymes (EC 2.7.1.117) (MLCK) [Co
395 (Q8N8V4) Harmonin-interacting ankyrin-repeat containing protein (Harp)
396 (Q8N1I0) Dedicator of cytokinesis protein 4
397 (P08572) Collagen alpha 2(IV) chain precursor
398 (Q9UPT6) C-jun-amino-terminal kinase interacting protein 3 (JNK-interacting protein 3) (JIP-3) (JNK
399 (P34931) Heat shock 70 kDa protein 1L (Heat shock 70 kDa protein 1-like) (Heat shock 70 kDa protein
400 (Q96DT5) Ciliary dynein heavy chain 11 (Axonemal beta dynein heavy chain 11)
401 (O95935) T-box transcription factor TBX18 (T-box protein 18)
402 (Q75N90) Fibrillin 3 precursor
403 (Q9H8G1) Zinc finger protein 430
404 (Q9H5X1) Hypothetical UPF0195 protein FLJ22875
405 (P59531) Putative Taste receptor type 2 member 12 (T2R12) (Taste receptor type 2 member 26) (T2R26)
406 (Q8WVM8) Sec1 family domain containing protein 1 (Syntaxin binding protein 1-like 2) (Vesicle trans
407 (P11055) Myosin heavy chain, fast skeletal muscle, embryonic (Muscle embryonic myosin heavy chain)
408 (P39193) Alu subfamily SP sequence contamination warning entry
409 (Q92502) StAR-related lipid transfer protein 8 (StARD8) (START domain-containing protein 8)
410 (Q96SQ9) Cytochrome P450 2S1 (EC 1.14.14.1) (CYPIIS1) (UNQ891/PRO1906)
411 (Q9NUA8) Hypothetical zinc finger protein KIAA0478
412 (Q05586) Glutamate [NMDA] receptor subunit zeta 1 precursor (N-methyl-D-aspartate receptor subunit
413 (O15442) Adult brain protein 239 (239AB)
414 (O75071) Protein KIAA0494
415 (P07332) Proto-oncogene tyrosine-protein kinase Fes/Fps (EC 2.7.1.112) (C-Fes)
416 (Q9H9S5) Fukutin related protein (EC 2.-.-.-)
417 (P48995) Short transient receptor potential channel 1 (TrpC1) (TRP-1 protein)
418 (O95183) Vesicle-associated membrane protein 5 (VAMP-5) (Myobrevin) (HSPC191)
419 (Q9UM47) Neurogenic locus notch homolog protein 3 precursor (Notch 3)
420 (Q92805) Golgi autoantigen, golgin subfamily A member 1 (Golgin-97)
421 (Q06203) Amidophosphoribosyltransferase precursor (EC 2.4.2.14) (Glutamine phosphoribosylpyrophosph
422 (Q92616) GCN1-like protein 1 (HsGCN1)
423 (Q9NZR2) Low-density lipoprotein receptor-related protein 1B precursor (Low-density lipoprotein rec
424 (Q9HD67) Myosin X
425 (Q96M32) Putative adenylate kinase 7 (EC 2.7.4.3)
426 (P43220) Glucagon-like peptide 1 receptor precursor (GLP-1 receptor) (GLP-1-R) (GLP-1R)
427 (Q05996) Zona pellucida sperm-binding protein 2 precursor (Zona pellucida glycoprotein ZP2) (Zona p
428 (P47775) Probable G-protein coupled receptor 12
429 (O15084) Ankyrin repeat domain protein 28
430 (P49767) Vascular endothelial growth factor C precursor (VEGF-C) (Vascular endothelial growth facto
431 (P54803) Galactocerebrosidase precursor (EC 3.2.1.46) (GALCERase) (Galactosylceramidase) (Galactosy
432 (Q9NUE0) Zinc finger DHHC domain containing protein 18
433 (Q9P032) UPF0240 protein C6orf66 (HSPC125) (My013 protein)
434 (Q9UPV9) 106 kDa O-GlcNAc transferase-interacting protein
435 (Q14161) ARF GTPase-activating protein GIT2 (G protein-coupled receptor kinase-interactor 2) (GRK-i
436 (Q15417) Calponin-3 (Calponin, acidic isoform)
437 (Q13155) Multisynthetase complex auxiliary component p38 (JTV-1 protein) (PRO0992)
438 (Q14651) I-plastin (Intestine-specific plastin)

439 (O00482) Orphan nuclear receptor NR5A2 (Alpha-1-fetoprotein transcription factor) (Hepatocytic tran
440 (P54259) Atrophin-1 (Dentatorubral-pallidoluysian atrophy protein)
441 (Q96T68) Probable histone-lysine N-methyltransferase, H3 lysine-9 specific (EC 2.1.1.43) (Histone H
442 (O14511) Pro-neuregulin-2 precursor (Pro-NRG2) [Contains: Neuregulin-2 (NRG-2) (Neural-and thymus-d
443 (Q9BXL7) Caspase recruitment domain protein 11 (CARD-containing MAGUK protein 3) (Carma 1)
444 (Q14147) Probable ATP-dependent helicase DHX34 (DEAH-box protein 34)
445 (Q92610) Zinc finger protein 592
446 (Q96PY6) Serine/threonine-protein kinase Nek1 (EC 2.7.1.37) (NimA-related protein kinase 1) (NY-REN
447 (Q9P0W8) Spermatogenesis associated protein 7 (Spermatogenesis associated protein HSD3) (HSD-3.1)
448 (P51991) Heterogeneous nuclear ribonucleoprotein A3 (hnRNP A3)
449 (Q9H2K2) Tankyrase 2 (EC 2.4.2.30) (TANK2) (Tankyrase II) (TNKS-2) (TRF1-interacting ankyrin-relate
450 (Q9P2M4) TBC1 domain family member 14
451 (Q9ULT8) HECT domain containing protein 1 (Fragment)
452 (O43399) Tumor protein D54 (hD54) (D52-like 2)
453 (P08908) 5-hydroxytryptamine 1A receptor (5-HT-1A) (Serotonin receptor 1A) (5-HT1A) (G-21)
454 (Q9H2U2) Inorganic pyrophosphatase 2, mitochondrial precursor (EC 3.6.1.1) (PPase 2) (Pyrophosphata
455 (Q9UL18) Eukaryotic translation initiation factor 2C 1 (eIF2C 1) (eIF-2C 1) (Putative RNA-binding p
456 (Q9UBM7) 7-dehydrocholesterol reductase (EC 1.3.1.21) (7-DHC reductase) (Sterol delta-7-reductase)
457 (Q13643) Skeletal muscle LIM-protein 2 (SLIM 2) (Four and a half LIM domains protein 3) (FHL-3)
458 (P98161) Polycystin 1 precursor (Autosomal dominant polycystic kidney disease protein 1)
459 (O94956) Solute carrier organic anion transporter family, member 2B1 (Solute carrier family 21, mem
460 (Q8NGA2) Olfactory receptor 7A2
461 (O94763) RNA polymerase II subunit 5-mediating protein (RPB5-mediating protein)
462 (Q99856) AT-rich interactive domain-containing protein 3A (ARID domain-containing protein 3A) (B-ce
463 (P61769) Beta-2-microglobulin precursor (HDCMA22P)
464 (Q9UQR0) Sex comb on midleg-like protein 2
465 (P21817) Ryanodine receptor 1 (Skeletal muscle-type ryanodine receptor) (RyR1) (RYR-1) (Skeletal mu
466 (Q8TE04) Pantothenate kinase 1 (EC 2.7.1.33) (Pantothenic acid kinase 1) (hPanK1) (hPanK)
467 (Q9BZE4) Nucleolar GTP-binding protein 1 (Chronic renal failure gene protein) (GTP-binding protein
468 (P57071) PR-domain zinc finger protein 15 (Zinc finger protein 298)
469 (Q8N4C6) Ninein (hNinein)
470 (Q9UNA1) Rho-GTPase-activating protein 26 (Oligophrenin-1 like protein) (GTPase regulator associate
471 (P35916) Vascular endothelial growth factor receptor 3 precursor (EC 2.7.1.112) (VEGFR-3) (Tyrosine
472 (O43493) Trans-Golgi network integral membrane protein 2 precursor (Trans-Golgi network protein TGN
473 (Q92598) Heat-shock protein 105 kDa (Heat shock 110 kDa protein) (Antigen NY-CO-25)
474 (Q13459) Myosin IXb (Unconventional myosin-9b)
475 (Q8N3T1) Polypeptide N-acetylgalactosaminyltransferase-like protein 2 (EC 2.4.1.41) (Protein-UDP ac
476 (Q13573) Nuclear protein SkiP (Ski-interacting protein) (SNW1 protein) (Nuclear receptor coactivato
477 (P16118) 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (6PF-2-K/Fru-2,6-P2ASE liver isozyme
478 (P13688) Carcinoembryonic antigen-related cell adhesion molecule 1 precursor (Biliary glycoprotein
479 (Q15772) Aortic preferentially expressed protein 1 (APEG-1)
480 (P83436) Conserved oligomeric Golgi complex component 7
481 (Q15648) Peroxisome proliferator-activated receptor binding protein (PBP) (PPAR binding protein) (T
482 (Q00722) 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta 2 (EC 3.1.4.11) (Phosphoino
483 (Q13435) Splicing factor 3B subunit 2 (Spliceosome associated protein 145) (SAP 145) (SF3b150) (Pre
484 (O15297) Protein phosphatase 2C delta isoform (EC 3.1.3.16) (PP2C-delta) (p53-induced protein phosp
485 (Q9H2T7) Ran-binding protein 17
486 (Q9C0D0) Phosphatase and actin regulator 1
487 (Q15596) Nuclear receptor coactivator 2 (NCoA-2) (Transcriptional intermediary factor 2)
488 (Q9Y252) RING finger protein 6 (RING-H2 protein)
489 (P42356) Phosphatidylinositol 4-kinase alpha (EC 2.7.1.67) (PI4-kinase) (PtdIns-4-kinase) (PI4K-alp
490 (Q8IUD2) ERC protein 1 (ELKS protein)
491 (P00367) Glutamate dehydrogenase 1, mitochondrial precursor (EC 1.4.1.3) (GDH)
492 (P49641) Alpha-mannosidase IIx (EC 3.2.1.114) (Mannosyl-oligosaccharide 1,3-1,6-alpha-mannosidase)
493 (Q9Y2X9) Zinc finger protein 281 (Zinc finger DNA binding protein 99) (Transcription factor ZBP-99)
494 (O95210) Genethonin 1 (GENX-3414)
495 (O75151) PHD finger protein 2 (GRC5)
496 (P78563) Double-stranded RNA-specific editase 1 (EC 3.5.-.-) (dsRNA adenosine deaminase) (RNA editi
497 (P62910) 60S ribosomal protein L32 (PP9932)
498 (P08621) U1 small nuclear ribonucleoprotein 70 kDa (U1 snRNP 70 kDa) (snRNP70) (U1-70K)
499 (Q9H2G9) Golgin 45 (Basic leucine zipper nuclear factor 1) (JEM-1) (p45 basic leucine-zipper nuclea
500 (Q99460) 26S proteasome non-ATPase regulatory subunit 1 (26S proteasome regulatory subunit RPN2) (2